The role of haem oxygenase in ischaemic preconditioning on the intestinal microcirculation following ischaemia reperfusion injury of the intestine

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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solemnly and sincerely declare, in relation to the thesis entitled:

The role of haeme oxygenase in ischaemic preconditioning on the intestinal microcirculation in ischaemia reperfusion injury of the intestine

(a) That work was done by me personally

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Dedication

I have been very fortunate to have an extremely supportive family. I dedicate this thesis to my parents Shahul Hameed and Jamila, who built the foundations upon which I based my values and judgement. I also dedicate this thesis to my wife Femina and my two wonderful boys Faris and Tarik, while I thought I was taking care of them, they were taking care of me.

I also dedicate this thesis to my late friend and post-doctoral colleague Wenxuan Yang who offered his intellectual and personal support through out the course of my study and without whose help this thesis would not have been possible. I miss him a lot.

Abstract

Intestinal ischaemia reperfusion (IR) injury is associated with various clinical conditions such as hypovolaemic shock, strangulation-obstruction, cardiovascular surgery, abdominal aortic surgery and small bowel transplantation. IR injury of the intestine is a systemic phenomenon resulting in bacterial translocation, endotoxaemia, acute respiratory distress syndrome and acute hepatic injury culminating in multiple organ failure.

It has been hypothesised that ischaemic preconditioning may protect against IR injury of the intestine through haem oxygenase formation. This thesis (study) investigated the relationship of haem oxygenase metabolism with intestinal microvascular perfusion, tissue oxygenation and function with ischaemic preconditioning of the intestine in an experimental model of ischaemia reperfusion injury of the small intestine.

Male Sprague Dawley rats (250-300 grams) were subjected to 30 mins of ischaemia by clamping of superior mesenteric artery followed by 2 hrs (early phase) or 24 hrs (late phase) of reperfusion. Ischaemic preconditioning was performed with 10 min ischaemia and 10 min reperfusion before the sustained ischaemia. Pyrrolidine dithiocarbamate (PDTC) or Zinc Protoporrphyrrin (ZnPP) were administered to stimulate or block heme oxygenase synthesis. The study demonstrated that ischaemic preconditioning resulted in significantly improvement in intestinal microvascular perfusion, tissue oxygenation as well as decreased leukocyte-endothelial interactions and intestinal and pulmonary injury following both early and late phases of IR injury.

The preconditioning effect was associated with significantly increased haem oxygenase production suggested by intestinal tissue haeme oxygenase levels as demonstrated by haem oxygenase assays and western blotting. PDTC treatment reproduced the protective effect of ischaemic preconditioning. Haem oxygenase inhibition with ZnPP antagonized the protective effect of ischaemic preconditioning.

This thesis has shown that the protective effect of intestinal ischaemic preconditioning against both early and late phases of IR injury is associated with increased haem oxygenase production. These data may have important implications in intestinal surgery and transplantation and may lead to the development of pharmacological strategies for protecting the intestine from ischaemic injury.

Acknowledgements

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List of abbreviations

ADP Adenosine diphosphate

ALT Alanine aminotransferase

AMP Adenosine monophosphate

AST Aspartate aminotransferase

(ALT) Alanine transaminase

ATP Adenosine triphosphate

CCD charged couple device

Cyt Ox Cu_A: Cytochrome oxidase Cu_A redox changes

eNOS endothelial nitric oxide synthase

FITC fluorescein isothiocyanate

Hb Deoxyhaemoglobin

HbO_2 Oxyhaemoglobin

HR Heart rate

HO Haem Oxygenase

iNOS inducible nitric oxide synthase

IPC Ischaemic preconditioning

IR Ischaemia reperfusion

IVFM Intravital fluoromicroscopy

LDF Laser Doppler flowmeter

MABP Mean arterial blood pressure

MCD Mean Capillary diameter

MPI Mucosal Perfusion index

L-NAME Nω-Nitro-L-arginine methyl ester

NIRS Near infrared spectroscopy

NO Nitric oxide

PDTC Pyrrolidine dithiocarbamate

PVF portal venous flow

RBC red blood cell

SaO_2 Oxygen saturation

XO xanthine oxidase

ZnPP Zinc Protoporphyrin

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Chapter 1 Literature review: The role of Microcirculation in Ischaemia Reperfusion Injury of the Intestine

1.1 Anatomical and Physiological Considerations

The entire gastrointestinal system is supplied by three direct branches of the aorta: the coeliac artery, the superior mesenteric artery, and the inferior mesenteric artery (Geboes *et al.*, 2001). The coeliac artery supplies blood flow to the stomach, liver, and spleen, while the superior mesenteric artery, the largest single branch of the abdominal aorta, supplies the entire small intestine, proximal portions of the large intestine and the pancreas. The inferior mesenteric artery supplies blood flow to the distal portion of the large intestine(Geboes *et al.*, 2001).

The small intestine is composed of three layers namely, the mucosa, submucosa and muscularis. Each of the major functions of the small intestine- absorption, secretion and motility is mainly localised to one of the three layers. Absorption is subserved by the large surface area created by the architecture of the mucosa with its abundance of villi and microvilli. The secretory function is mainly localised to the submucosa and basal region of the mucosa i.e. in the glands located at the base of the villi and lining the crypts. Lastly, the intestinal movements, both propulsive and non-propulsive are dependent on the contraction and relaxation of the longitudinal and circular smooth muscles of the muscularis(Granger *et al.*, 1980).

The superior mesenteric artery divides into arcade branches which supply the intestine through the mesentery. They enter the serosa and arborize into several branches. Some of these branches converge in the anti-mesenteric border where many anastomoses are formed. In their course towards the anti-mesenteric border these blood vessels give rise to numerous branches that form the mural trunks of the serosa. From each villus, one or more vessels are formed which course outward from the villus to

eventually joins the veins of the glandular region before entering the submucosa where they merge with the veins draining the submucosal plexus. These vessels continue on through the muscularis layer and serosa, and then merge with other vessels, before draining into the portal vein via the superior mesenteric vein (Lanciault and Jacobson, 1976). Blood flow to the mucosa-submucosal layer normally accounts for 80% of the total intestinal flow. The high proportion of blood flow to the mucosa-submucosa presumably reflects the higher metabolic demand in these layers of the small intestine (Bohlen, 1998).

The term 'microcirculation' refers to the vascular network and includes the resistance vessels, the pre-capillary sphincters, the true capillaries, the post-capillary sphincters and the venulae with the flow of blood through this network (Uhlmann *et al.*, 1999). The resistance vessels of the intestine regulate the arterial blood inflow pressure to the intestine and the proportion of the cardiac output. The pre and the post-capillary sphincters regulate the mean hydrostatic capillary pressure, a variable which is crucial in the determination of the direction and the magnitude of net movement of fluid across the capillary. The pre-capillary sphincter determines the number of capillaries that are open at a time point. The vascular bed of the intestine reduces its vascular resistance to blood flow following a decrease in perfusion pressure. This autoregulation of blood flow is effective over a wide pressure range. Autoregulation is described as an intrinsic ability of an organ to maintain a constant blood flow in the face of a fluctuating arterial pressure (Johnson, 1960). The site of autoregulation in the intestine has been localised to the pre-capillary resistance vessels. As the perfusion pressure falls, flow is redistributed among the various layers of the intestine, with an increase in flow to the more metabolically active superficial mucosa.

The intestinal microcirculation has unique characteristic features that are essential in the understanding of the intestinal reaction to ischaemia. The intestinal vascular bed has a high capillary density and high capillary permeability than other vascular circuits, excluding the hepatic sinusoids. The venous blood pressure and mean hydrostatic capillary pressure is higher in the intestine than other vascular beds. Hence, the intestinal vascular bed is a risk of losing considerable amount of fluids when the capillary pressure becomes elevated. The homeostatic mechanisms aim to maintain these pressures constant. These delicate mechanisms may be altered in ischaemia reperfusion (IR) injury of the intestine.

1.2 Intestinal Ischaemia Reperfusion Injury

Ischaemia reperfusion (IR) injury of the intestine is an important factor associated with a high morbidity and mortality in both surgical and trauma patients (Koike *et al.*, 1993). It is of importance in situations such as the interruption of blood flow to the gut as in abdominal aortic aneurysm surgery, cardiopulmonary bypass, strangulated hernias, neonatal necrotising enterocolitis and intestinal transplantation (Collard and Gelman, 2001). IR injury also occurs in septic and hypovolemic shock (Moore *et al.*, 1994;Swank and Deitch, 1996).

Interruption of blood supply results in ischemic injury which rapidly damages metabolically active tissues. Paradoxically, restoration of blood flow to the ischemic tissue initiates a cascade of events that may lead to additional cell injury known as reperfusion injury. This reperfusion damage frequently exceeds the original ischemic insult (Stallion *et al.*, 2002). On restoration of the blood supply, the molecular and

biochemical changes that occur during ischaemia predispose to free radical mediated damage. The reduction of blood supply results in damage of the intestinal mucosa.

Among the internal organs, the intestine is probably the most sensitive to IR injury (Granger *et al.*, 1986;Yamamoto *et al.*, 2001). The intestine is composed of labile cells that are easily injured by episodes of ischaemia. Subsequent reperfusion of the intestine results in further damage of the mucosa (Kong *et al.*, 1998). It has been shown that the enterocytes that are located at the tips of the villi are more sensitive to the effect of ischaemia. This was attributed to their location at the end distribution of a central arteriole which leads to lower oxygen tension compared to the crypt (Kong *et al.*, 1998;Takeyoshi *et al.*, 1996). However a recent study in a rat model demonstrated that the sensitivity of an enterocyte is dependent on the state of its differentiation (Hinnebusch *et al.*, 2002).

There is substantial evidence that the mucosa of the intestine becomes the site for the production of various acute-phase proteins (Molmenti *et al.*, 1993;Wang *et al.*, 1998), gut hormones (Zamir *et al.*, 1992)and cytokines (Mester *et al.*, 1993;Meyer *et al.*, 1995).These influence not only the intestine but may also effect the function and integrity of distant organs. The initial site of abnormality in ischaemia has been emphasised on the cellular mitochondria, which is particularly important in producing adenosine triphosphate (ATP) for organ recovery (Jassem *et al.*, 2002). The damage, however, is dramatically magnified by a large number of events, such as oxygen free radical formation, release of iron storage, damage of the microvasculature of IR organs, inflammatory cytokines, complement activation and neutrophil infiltration at the site of injury (Carden and Granger, 2000). The enterocyte, in particular has been shown to

produce increased amounts of cytokines during sepsis, endotoxemia, hemorrhagic shock and trauma (Ogle *et al.*, 1994) .

1.3 Consequences of Intestinal Ischaemia Reperfusion Injury

1.3.1 Alteration of absorptive function of intestine

Several studies have demonstrated an alteration of absorptive function of the intestine following IR injury of the intestine (Kuenzler *et al.*, 2002b;Prasad *et al.*, 2001;Rajeevprasad *et al.*, 2000) . This could lead to deficient absorption of nutrients. In severe cases such as infarction of the bowel, short-bowel syndrome can occur (Scolapio and Fleming, 1998) . Sileri *et al.* demonstrated in a rat model that IR injury of the intestine causes both acute and chronic alterations of the intestinal absorptive function, which was associated with significant mortality (Sileri *et al.*, 2002).

1.3.2 Bacterial translocation

Bacterial translocation is the passage of viable bacteria from the gastrointestinal tract through the epithelial mucosa to the extraintestinal sites such as mesenteric lymph nodes, liver and spleen (Van Leeuwen *et al.*, 1994). Subsequently the bacteria can disseminate throughout the body producing sepsis, shock or multiple organ failure (MOF). Bacterial translocation has been reported to occur in 44% of paediatric patients undergoing small bowel transplantation (Cicalese *et al.*, 2001). The increase in intestinal hyperpermeabilty occurring in IR injury of the intestine is one of the factors causing bacterial translocation (Aksoyek *et al.*, 2002;Iijima *et al.*, 1997;Xia *et al.*, 2002) . The other factors are overgrowth of bacteria in the small intestine and differences in host immune defences (Berg, 1999).

1.3.3 Injury to distant organs

IR injury to the intestine results in production of molecules such as hydrogen peroxide, superoxide and inflammatory cytokines that may harm distant organs. This leads to the development of Systemic Inflammatory Response Syndrome (SIRS) which can progress to MOF (Ceppa *et al.*, 2003). Intestinal IR injury also causes pulmonary infiltration of neutrophils, which contributes to the development of acute respiratory distress syndrome (ARDS) (Koksoy *et al.*, 2001;Xiao *et al.*, 1997). Hence, the gut has been referred to as 'the motor of MOF' (Marshall, 1998).

1.4 Mediators Involved in IRI of the Intestine

1.4.1 Xanthine oxidase & oxygen free radicals

Xanthine oxidase (XO) is a highly versatile enzyme which plays an important role in the catabolism of purines. The intestine is the richest source which is the initial source of free radicals that can cause further tissue damage (Parks *et al.*, 1988). In mammals xanthine dehydrogenase (XDH) can be converted to XO either reversibly or irreversibly (Meneshian and Bulkley, 2002). During the ischemic period, cellular ATP is catabolised to yield hypoxanthine (Younes *et al.*, 1984). The hypoxic stress also triggers the conversion of XDH to the oxygen radical-producing XO. ATP depletion results in loss of ATP-dependent ion channel regulation, producing passive ion shifts across cell membranes: K^+ and Mg^{++} diffuse out, but Na^+, Ca^{++} and H_2O diffuse in causing cell swelling. Increased cellular Ca^{++} is harmful, one of its important consequences being the activation of a calcium-dependent protease, which cleaves XDH to XO. During reperfusion, molecular oxygen is re-introduced into the tissue where it reacts with hypoxanthine and XO to produce a burst of oxygen free radicals- superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Harrison, 2002) (Fig 1.1).

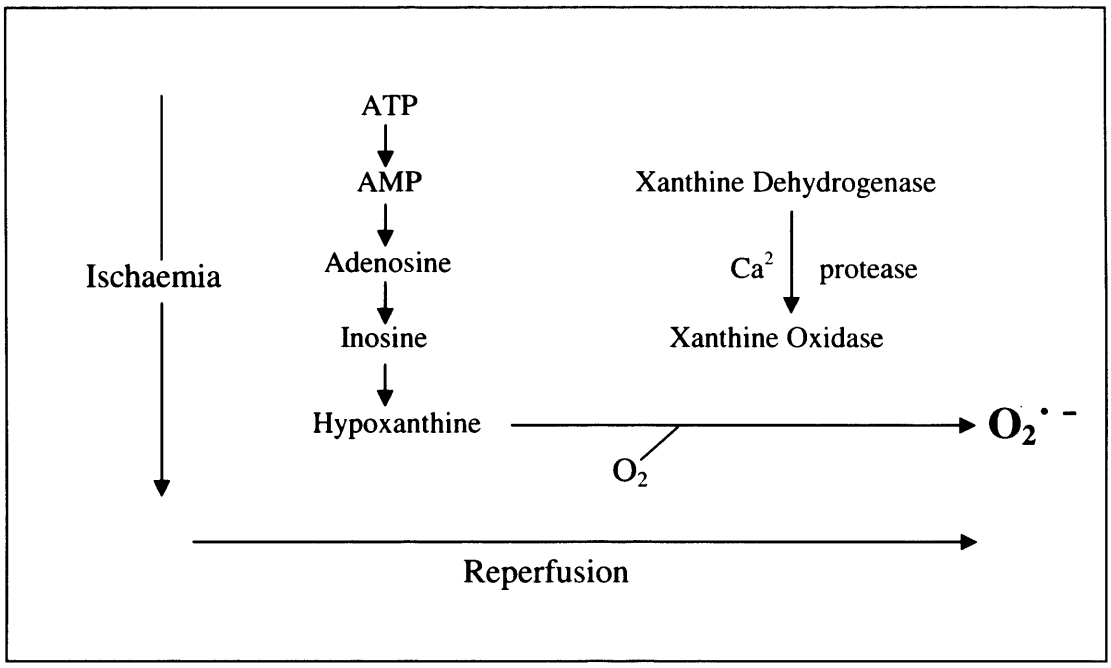


Figure 1.1. Mechanism of xanthine oxidase mediated free radical injury.

Modified from Granger (Granger et. al, 1986)

Under physiological conditions, the damaging effects of $O_2^{\cdot -}$ are prevented by superoxide dismutase (SOD) , which converts $O_2^{\cdot -}$ to H_2O_2 (Harrison, 2002). However, during reperfusion of ischemic tissues, these natural defenses may be overcome. $O_2^{\cdot -}$ itself is a relatively low energy radical, but it is responsible for production of the highly reactive and damaging hydroxyl radical ($OH^{\bullet}$) .Typically the $OH^{\bullet}$ causes biological damage by stimulating the free chain reaction known as lipid peroxidation, in which $OH^{\bullet}$ attacks the fatty acid side chains of the membrane phospholipids and causes organelle and cell disruption (Halliwell and Gutteridge, 1999).

1.4.2 Endothelins

Endothelins (ETs) are potent peptide vasoconstictors derived from the vascular endothelium (Ozel *et al.*, 2001). Among the three active isoforms (ET-1, ET-2 and ET-3) , ET-1 is the most powerful endogenous vasoconstrictor substance known to date (Inoue *et al.*, 1989;Yanagisawa *et al.*, 1988).The vasoconstrictive effects of ET-1 are predominantly mediated via ET-A receptors present on vascular smooth muscle cells .The ET-B receptors mediate vasoconstriction ($ET\text{-}B_2$) and vasodilatation ($ET\text{-}B_1$) (Clozel *et al.*, 1992). ET receptor antagonists provide significant protection against IR injury of the intestine (Anadol *et al.*, 1998;Andrasi *et al.*, 2002;Buyukgebiz *et al.*, 1994;Oktar *et al.*, 2002;Wolfard *et al.*, 2002). The major beneficial effects of ET inhibition include a decrease in mucosal lesions, an increase in blood flow, an increase in mucosal ATP and a decrease in leukocyte adhesion (Oktar *et al.*, 2000).

1.4.3 Heat shock proteins

Heat Shock Proteins (HSP) are intracellular stress proteins that have been shown to accumulate after ischaemia (Tsuruma *et al.*, 1996b).The concept of sub-lethal whole body hyperthermia conferring tolerance to ischaemia and endotoxin exposure is known

as hyperthermic preconditioning and has been associated with HSP accumulation (Hotchkiss *et al.*, 1993;Ryan *et al.*, 1992). In the rat intestine, tolerance to ischemic injury has been associated with the production of various inducible HSP: HSP-70 (Fleming *et al.*, 2002), HSP-72 (Stojadinovic *et al.*, 1995) and HSP-73 (Tsuruma *et al.*, 1999). The exact mechanism whereby HSPs confer protection of the intestine against IR injury is not known. Stojadinovic and co-workers showed that a probable mechanism involves the inhibition of leukotriene-B_4 production and subsequent prevention of neutrophilic activation and chemotaxis (Stojadinovic *et al.*, 1995).

1.4.4 Polymorphonuclear neutrophils (PMN)

There is a growing body of evidence that incriminates PMNs in the pathophysiology of IR injury (Hayward and Lefer, 1998;Hierholzer *et al.*, 1999;Cooper *et al.*, 2004;Mbachu *et al.*, 2004). In a model of small bowel transplantation, Massberg et al showed the manifestation of IR injury is primarily due to the leukocyte-endothelial cell interactions in the submucosal venules of the transplanted intestine (Massberg *et al.*, 1998).

PMNs depleted blood in the reperfusate has been shown to ameliorate IR injury in the human small bowel (Sisley *et al.*, 1994). Intravital microscopic studies of tissues exposed to IR injury reveal an acute inflammatory response that is characterised by increased adhesion and emigration of PMNs in post capillary venules and enhanced protein efflux (Granger and Korthuis, 1995). Indirectly, the assessment of neutrophil migration into the small intestine is performed by measuring myeloperoxidase (MPO) activity in the small intestine(Naito *et al.*, 2002).

The emigration of PMNs from the post capillary venules to areas of inflammation is a complex and a highly coordinated three-step process namely rolling, adhesion and extravasation (Grisham *et al.*, 1998)(Fig 1.2).

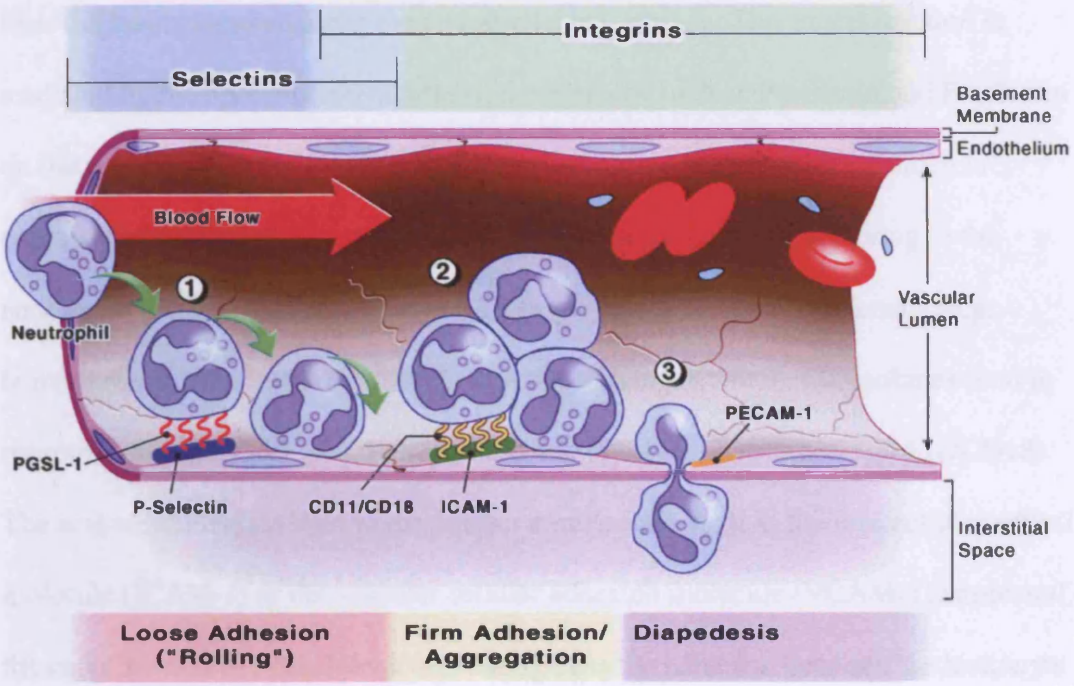


Figure 1.2. Sequence of events in emigration of neutrophils.

First, the endothelial Selectins are upregulated and interact with the oligosaccharides on the surface of leukocytes. This then brings the leukocyte into close proximity of chemoattractants such as chemokines which then activates the integrins. Integrins (CD11/CD18) binds to the endothelial receptors (ICAM-1) resulting in strong adhesion followed by extravasation of the neutrophils. Reproduced from Collard and Gelman(Collard and Gelman, 2001).

First the leukocytes roll along the endothelial cell surface. This initial reaction is mediated by the upregulation of adhesion molecules such as P-selectin and E-selectin on the surface of the endothelium and their interaction with L-selectin which is expressed constitutively by the leukocytes. This weak leukocyte tethering to the endothelium brings the leukocyte into close proximity of chemoattractants (e.g., leukotriene B_4, C5a and PAF) and chemokines (Granger, 1988). Chemokines bind to receptors on leukocytes to activate β_2 integrins (CD11a, CD11b and CD 11c/CD18). The activated integrins bind to the adhesion molecules such as the intercellular adhesion molecule (ICAM-1) or the vascular cellular adhesion molecule (VCAM-1) expressed on the endothelial cells. This interaction strengthens the adhesion between the leukocyte and the endothelial cells, thereby allowing extravasation of the leukocyte out of the circulation into the interstitium (Panes *et al.*, 1999). Riaz *et al.* showed that following IR injury of the mouse intestine, both P&E selectins were expressed (Riaz *et al.*, 2002a). Pre-treatment with anti-P selectin antibody reduced PMNs rolling and adhesion, thereby attenuating the injury (Riaz *et al.*, 2002a). PMNs can cause tissue damage by several ways including secretion of proteolytic enzymes such as elastase from cytoplasmic granules (Carden *et al.*, 1990), production of free radicals via the respiratory burst (Weiss, 1989) and by physical impairment of the microcirculation and thereby extension of ischaemia (Bagge *et al.*, 1980). Nalini and colleagues showed that it is the PMNs and not XO that are the initial source of free radicals in a rat model of IR injury of the intestine (Nalini *et al.*, 1993).

1.4.5 Nitric Oxide (NO)

NO is a free radical and a highly reactive substance. NO is synthesised from L-arginine by a family of enzymes known as NO synthases (NOS). Three isoforms of NOS have identified and cloned namely: endothelial NOS (eNOS) , neuronal NOS (nNOS) and

inducible NOS (iNOS) (Albrecht *et al.*, 2003). Whereas eNOS and nNOS are constitutively expressed, iNOS is only expressed in response to cytokines and growth factors. nNOS is the predominant NOS in the normal intestine and this suppresses the expression of iNOS (Qu *et al.*, 2001). The possible sources of NO in the intestine include intrinsic intestinal tissue (epithelium, mast cells, smooth muscle and neural plexus) and the leukocytes (PMNs and monocytes) (Salzman, 1995).

The role of NO in IR injury is still a matter of considerable controversy. NO plays a dichotomous role both as a cytotoxic and cytoprotective molecule in intestinal IR injury. Whereas, inhibition of NO causes tissue dysfunction in certain models of IR injury, inhibition of NO provides benefit in others. The constitutive forms of NO synthase (nNOS and eNOS) are critical to normal physiology and inhibition of these enzymes cause tissue damage (Kubes and McCafferty, 2000). The induction of iNOS produce large amounts of NO and peroxynitrite leading to tissue injury and therefore specific inhibition of this enzyme is considered beneficial (Kubes and McCafferty, 2000). Additionally, iNOS knockout mice exhibited significant resistance to mucosal barrier dysfunction and bacterial translocation after intestinal IR injury, further supporting a role for iNOS as an important mediator of reperfusion injury in the intestine (Suzuki *et al.*, 2000). On the contrary, Cuzzocrea *et al* provided evidence that the production of peroxynitrate was not related to the induction of iNOS, whereas it was associated with the inhibition of eNOS (Cuzzocrea *et al.*, 1998). In a recent study, Wu *et al.* demonstrated that the formation of iNOS after IRI of the intestine enhanced mucosal apoptosis leading to IR injury of the intestine (Wu *et al.*, 2002). Virlos *et al* showed that pulmonary injury following intestinal IR injury was associated with decreased eNOS expression in the lung (Virlos *et al.*, 2003). Expression of iNOS occurred during the later stages of reperfusion, with overproduction of NO and

nitrosylation of protein tyrosine residues, thus aggravating pulmonary injury(Virlos *et al.*, 2003).

NO has many beneficial effects in the intestine such as scavenging of oxygen free radicals, maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, reduction of leukocyte adherence to the mesenteric endothelium, prevention of mast cell activation and inhibition of platelet aggregation (Wink *et al.*, 1993;Kosonen *et al.*, 1999;Gidday *et al.*, 1998;Guo *et al.*, 1996;Lefer and Lefer, 1996).

Under physiological conditions, NO will predominate in the microcirculation (Wink and Mitchell, 1998). However during the early phase of reperfusion in IR injury there is accumulation of O^{2-}. This would limit the accumulation of NO and prevent any of its beneficial effects. Hence there is a tendency for the PMNs to adhere to the endothelium and for the platelets to aggregate (Gauthier *et al.*, 1994).In the absence of NO, the O^{2-} produced will undergo spontaneous dismutation to form H_2O_2. The H_2O_2 formed in this way can promote the activation of Phospholipase A_2 (PLA2) and leads to the accumulation of pro-inflammatory mediators such as platelet activating factor (PAF) and leukotrienes (LTB_4) (Summarised in Fig 1.3). These factors can lead to activation of transcription factor nuclear factor kappa B ($NF-_KB$) which is a primary regulator of cytokine and adhesion molecule gene expression. Thus, the loss of endogenous NO production has disastrous consequences resulting in dysfunction of the endothelium and PMNs mediated tissue injury.

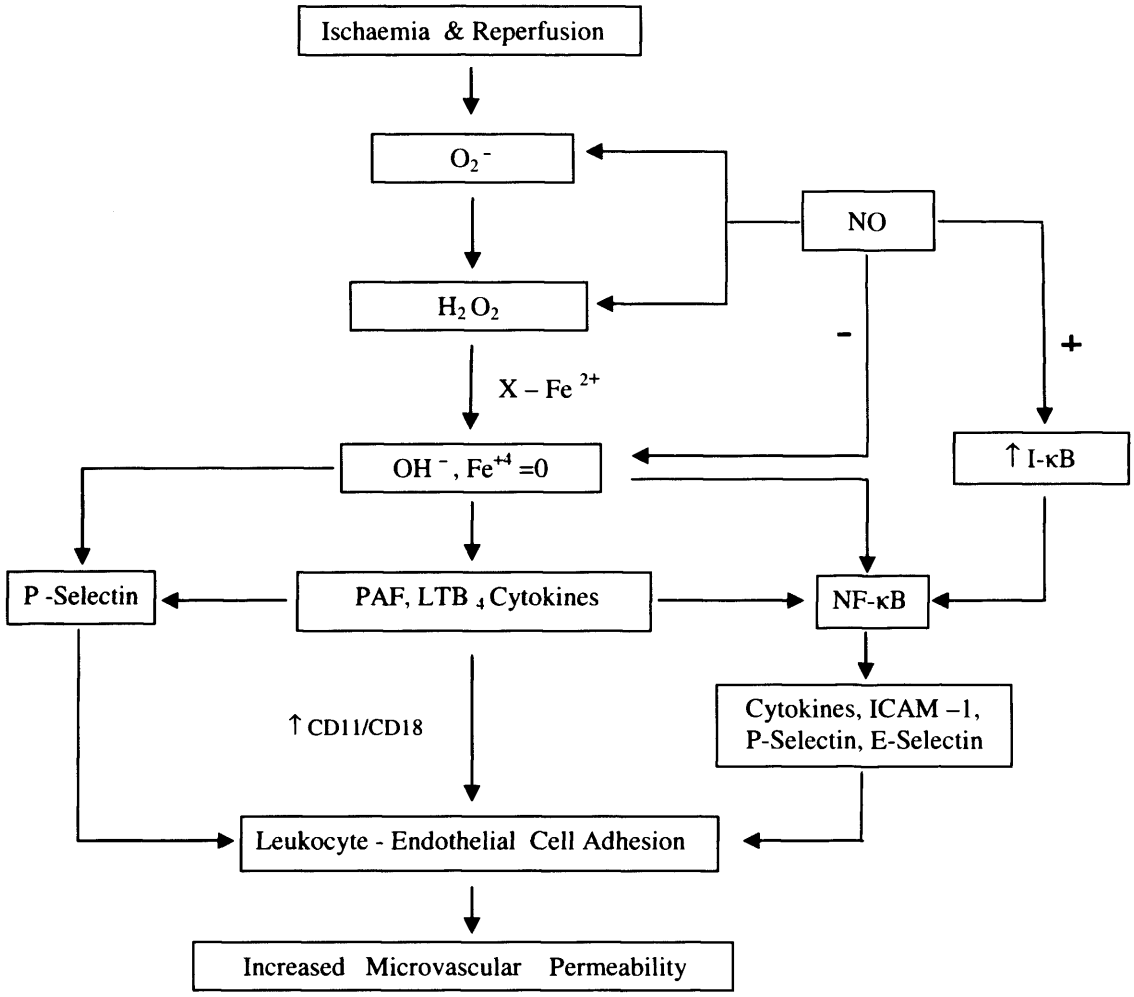


Figure 1.3. Role of nitric oxide in ischaemia – reperfusion injury.

1.4.6 Haem Oxygenase

HO-1 expression in various models of IR injury of the intestine has shown to offer cytoprotection during the cascade of events in IR injury (Ito *et al.*, 2003;Attuwaybi *et al.*, 2004;Nakao *et al.*, 2003;Squiers *et al.*, 1999). HO-1 is the rate-limiting enzyme in the conversion of heme into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (Fe^{2+}) (Choi and Alam, 1996). The HO-1 system is thought to play a pivotal role in the maintenance of antioxidant and oxidant homeostasis during cellular injury (Katori *et al.*, 2002b). Three isoforms of HO have so far been identified: inducible HO-1; constitutively expressed HO-2 and HO-3 which is related to HO-2, but is less well characterized. The HO-1 system exerts four major beneficial effects broadly: (a) antioxidant function; (b) maintenance of the microcirculation; (c) anti-apoptosis and (d) anti-inflammatory function (Katori *et al.*, 2002b). The antioxidant function relies on heme degradation, production of bilirubin (Stocker *et al.*, 1987) and the formation of ferritin via Fe^{2+} (Balla *et al.*, 1992). The production of CO with its vasodilatory and anti-platelet properties maintains the microcirculation and may be involved in the anti-apoptotic and cell arrest mechanisms (Katori *et al.*, 2002b). The HO-1 system exerts anti-inflammatory effects via modulation of endothelial adhesion molecules and chemoattractant factors (Willis *et al.*, 1996). Vachharajani *et al* provided evidence that HO-1 and biliverdin modulate leukocyte recruitment by altering the expression of P and E-selectins in the intestine, lungs, liver and kidneys (Vachharajani *et al.*, 2000). It has also been shown that HO-1 inhibits the NO system by downregulating iNOS, which contains a heme-iron moiety (Coito *et al.*, 2002).

Nakao et al showed that inhalation of CO improved graft function and survival in a rat model of small bowel transplantation (Nakao *et al.*, 2003). Exogenous administration of bilirubin has shown to reduce intestinal injury in rat models of IR injury (Ceran *et al.*,

2001;Hammerman *et al.*, 2002). Doxorubicin has been shown to induce HO-1 and attenuate lung injury following IRI of the intestine (Ito *et al.*, 2003). Squiers *et al* have shown that HO-1 overexpression in small bowel donors may decrease IR injury and improve survival of both the animal and the transplanted bowel (Squiers *et al.*, 1999). Recently Attuwaybi *et al* demonstrated that subcutaneous administration of hemin (HO-1 inducer) significantly reduced IR injury of the intestine (Attuwaybi *et al.*, 2004).

1.4.7 Pro-inflammatory Proteins

The precise chemical steps connecting the generation of free radicals and secretion of inflammatory mediators by the endothelial cells are not well understood. It appears that NO is a key factor involved in the synthesis of pro-inflammatory proteins (Anaya-Prado *et al.*, 2002).NO may be exerting its effects at the transcription level probably by the regulation of NF-$_K$B which regulates the expression of several genes that are involved in the process of IRI (Zingarelli *et al.*, 2003)(Fig 1.4). NF-$_K$B belongs to a family of dimeric transcription factor complexes which is normally found in the cytoplasm of unstimulated cells in their inactive forms i.e., as the p50/p65/I-$_K$B ternary complex or the p65/p105 inactive binary complex. Stimulation of cells with certain cytokines, oxidants or inflammatory mediators such as PAF or LTB_4 results in phosphorylation and ubiquitination of the I-$_K$B or p105 subunit (Siebenlist *et al.*, 1994;Collins *et al.*, 1995). This results in translocation of the subunit to the nucleus to activate transcription of its target genes. NF-$_K$B induces the expression of adhesion molecules (ICAM-1, VCAM-1, P-selectin, E-selectin), tumour necrosis factor (TNF- α and β), interleukins, chemokines and enzymes (inducible nitric oxide synthase and cyclooxygenase-2) (Barnes, 1997;Blackwell and Christman, 1997). It appears that in IR injury, reoxygenation is the key stimulus in the regulation of NF–$_K$B .By maintaining the interaction between I_KBα and NF–$_K$B, upregulation of adhesion molecules is inhibited

(Spiecker *et al.*, 1998). NO appears to prevent the formation of substances that activate NF–$_K$B as well as enhance the activity of proteins that inhibit NF–$_K$B. Previous studies suggest that NO may induce the denovo synthesis or stabilise I_KBα, thereby limiting NF–$_K$B activation (Peng *et al.*, 1995).

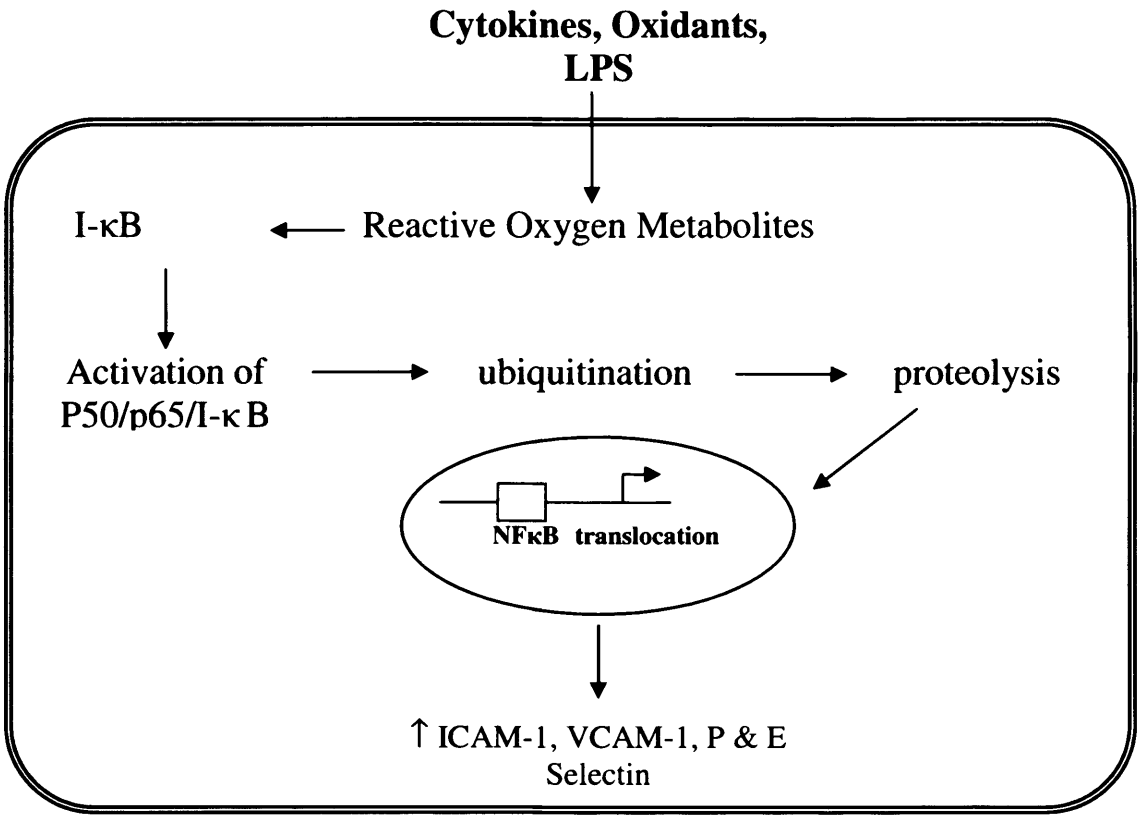


Figure 1.4. The relationship between oxidants and activation of NF–$_{K}$B.

The transcription factor NF–$_{K}$B is normally found in the cytoplasm of the cell in an inactive form as p50/p65/ I- $_{K}$B complex. Upon stimulation of the cells with cytokines, oxidants or bacterial products leads to an increase in reactive oxygen metabolites, which then activates I- $_{K}$B kinase. This kinase phosphorylates the inhibitory complex, which is then ubiquitinated. Following which proteolysis occurs by the action of the 26S proteasome complex and nuclear translocation of the active NF–$_{K}$B.

1.4.8 Complement

Several studies have suggested that the complement system is a major candidate in the pathogenesis of IR injury of the intestine (Austen Jr *et al.*, 2003;Heller *et al.*, 1999;Kimura *et al.*, 1998;Wada *et al.*, 2001).P-selectin has been shown to mediate IR injury of the intestine via complement(C5b-9) deposition in the intestinal mucosa (Gibbs *et al.*, 1996). Activation of the complement cascade leads to a series of potent inflammatory events including the expression of ICAM-1 gene and the up-regulation of cytokines such as TNF-α and interleukin (IL-1 α) (Wada *et al.*, 2001).In a recent study it has been shown by Montalto *et al.* that complement mediates up-regulation of intestinal iNOS and diminishes protein levels of mitochondrial SOD and the activity of Cu/Zn SOD (Montalto *et al.*, 2003).

1.4.9 Iron

During ischaemia, metabolites such as lactic acid accumulate and the intracellular pH falls. This decreases the stability of lysosomal membranes, activates lysosomal lytic enzymes, inhibits the binding of transitional metals such as iron to their carrier proteins (e.g. transferrin and ferritin) and results in increased free intracellular iron (Fe^{2+}) , which accelerates *in vivo* free radical formation in the following (Haber – Weiss) reaction: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + H^\bullet$ (Hernandez *et al.*, 1987).Using chelating agents of iron such as desferrioxamine has proved to be effective in reducing IRI because these agents prevent the Haber - Weiss reaction and hydroxyl radical formation (Lelli, Jr. *et al.*, 1993).

1.5 Protective Strategies against Ischaemia-Reperfusion Injury of the Intestine

The mechanisms of IR injury of the intestine and the ways to limit these have been under extensive research for almost a decade. Several therapeutic modalities have been successfully used to attenuate reperfusion injury in animal models of IR injury of the intestine. Potential strategies to overcome IR injury include several modalities: (a) ischaemic preconditioning (IPC) ; (b) antioxidants; (c) NO supplementation; (d) Anti-complement therapy; (e) anti-leukocyte therapy; (f) perfluorocarbons, (g) enteral feeding; (h) glutamine supplementation; and (i) glycine supplementation.

1.5.1 Ischaemic Preconditioning (IPC)

IPC refers to a phenomenon whereby exposure of a tissue to brief periods of ischaemia protects them from the deleterious effects of prolonged IR injury (Richard *et al.*, 1994). This fascinating phenomenon was first described in 1986 by Murry *et al.* in the heart of canines (Murry *et al.*, 1986). Subsequently beneficial effects have been demonstrated in the liver (Koti *et al.*, 2002a), skeletal muscle (Pang *et al.*, 1997), brain (Glazier *et al.*, 1994), spinal cord (Sakurai *et al.*, 1998), kidney (Turman and Bates, 1997), lung (Du *et al.*, 1996), retina (Li *et al.*, 2003) and intestine (Sola *et al.*, 2001;Davis *et al.*, 1999;Vlasov *et al.*, 2002;Aksoyek *et al.*, 2002) in various animal models. There is compelling evidence that IPC is beneficial in the human heart (Jenkins *et al.*, 1997) and the liver (Clavien *et al.*, 2003).

IPC of the intestine was first described by Hotter *et al.* in 1996 (Hotter *et al.*, 1996) and subsequent studies have confirmed this phenomenon (Table1.1). During this procedure, the small bowel is rendered ischaemic by placing an occlusion vascular clamp around the superior mesenteric artery. After an ischaemic interval of 5-20 minutes, the clamp is removed and the small intestine is reperfused for 5-15 minutes,

before the prolonged ischaemic insult. However, the ideal IPC protocol including the time of short ischaemia and number of cycle(s) is still not established. Some studies have reported that more than one cycle of ischaemia and reperfusion is required to achieve this beneficial effect (Unal *et al.*, 2003;McCallion *et al.*, 2000).

Table 1.1 A list of studies in which ischaemic preconditioning was performed against reperfusion injury in the intestine.

Author	Species	IPC Protocol	Mechanism	Outcome
Aksoyek S (Aksoyek *et al.*, 2002)	Rats	10 mins of ischaemia & 10 mins of reperfusion	↓iNOS expression	↓tissue damage & bacterial transloc
Cinel I(Cinel *et al.*, 2003)	Rats	10 mins of ischaemia	anti-apoptosis	↓number of apoptotic cells
Ferencz A (Ferencz *et al.*, 2002)	Dogs	4 cycles of 5 mins ischaemia & 10 mins of reperfusion	oxidative stress	↑GSH & SOD
McCallion K (McCallion *et al.*, 2000)	Rats	3 cycles of 2 min ischaemia & 5 min of reperfusion	↑adenosine	protects against mucosal barrier dysfunction
Sola A(Sola *et al.*, 2001)	Rats	10 mins of ischaemia & 5 mins of reperfusion	↑NO synthesis	↓tissue damage in this model of intestinal transplantation
Tamion F (Tamion *et al.*, 2002)	Rats	4 cycles of 1min ischaemia & 10 mins of reperfusion	expression of HO-1	↓fluid requirements, ↓pulmonary oedema, ↓lactate & TNF-α
Unal S(Unal *et al.*, 2003)	Rats	2 cycles of 5 min ischaemia & 5 min reperfusion	↓neutrophil infiltration, ↓oxidative stress and anti-apoptotic	↓MDA, ↓MPO, ↓tissue damage
Vlasov TD(Vlasov *et al.*, 2002)	Rats	10 mins of ischaemia & 10 mins of reperfusion	↑NO synthesis	↓tissue damage
Wu B(Wu *et al.*, 2004)	Rats	20 mins of ischaemia & 5 mins of reperfusion	anti-apoptosis	↓reactive oxygen species, ↓lipid peroxidation, ↓cytochrome c &↓casp 6

GSH, glutathione; HO-1, haem oxygenase-1; iNOS, inducible nitric oxide synthase; MDA*, malonyldialdehyde; MPO, myloperoxidase; NO, Nitric Oxide; SOD, superoxide dismutase.

The effects of IPC can be differentiated into two phases characterized by different time frames and mechanisms: (a) an early phase (early or classic preconditioning) that immediately follows the transient ischaemia and lasts 2–3 hours and (2) a late phase (late preconditioning), which begins 12–24 hours from the transient ischaemia and lasts for about 3–4 days (Cohen *et al.*, 2000;Bolli, 2000;Yellon and Dana, 2000) .Whereas early IPC occurs within minutes and involves the direct modulation of specific cell functions (Cohen *et al.*, 2000), the late phase requires the simultaneous activation of multiple stress-response genes with de novo synthesis of several proteins (Bolli, 2000). Despite these differences, both phases of preconditioning can be initiated by the same stimuli and share the same intracellular signal pathways to a certain extent (Bolli, 2000). Because of its sustained duration, late preconditioning might be particularly relevant for improving the results of small bowel transplantation. However, the present knowledge of the mechanisms responsible is quite preliminary. Recently, Ferencz *et al.* demonstrated that synthesis of proteins such as SOD and GSH was instrumental in the protection afforded by delayed IPC in a canine model of small bowel autotransplantation (Ferencz *et al.*, 2004).

The exact mechanism by which IPC confers protection in the intestine is unclear. Several mediators have been advocated to play a crucial role in this protective phenomenon including adenosine (Unal *et al.*, 2003;McCallion *et al.*, 2000), NO (Sola *et al.*, 2001), oxidative stress (Ferencz *et al.*, 2002), heme oxygenase 1 (HO-1)(Tamion *et al.*, 2002) and anti-apoptosis (Wu *et al.*, 2004).

Aksoyek *et al.* showed that IPC of the intestine decreases the translocation of bacteria from the intestine in a rat model of IRI (Aksoyek *et al.*, 2002). In a rat model of intestinal transplantation, Sola *et al.* identified a protective pathway initiated by IPC that

involves the activation of NO synthesis (Sola *et al.*, 2001) . This increase of NO synthesis was short-lived and detected at the end of preconditioning. The use of L-NAME eliminated this protection.

Davis *et al.* showed that IPC completely prevented the expression of P-selectin in the ischemic intestine and abolished the IRI induced leukocyte rolling (Davis *et al.*, 1999). These powerful anti-inflammatory actions of IPC were thought to be triggered by stimulation of adenosine A1 receptors during the period of preconditioning.

The protective effect of IPC has also been associated with a decrease of xanthine accumulation and a diminished conversion of XDH to XO (Sola *et al.*, 2000). There is evidence that IPC reduces apoptosis in the intestinal epithelial cells and this is attributed to significant expression of Bcl-2 (Cinel *et al.*, 2003).

Tamion *et al.* in a rat model of haemorrhagic shock followed by resuscitation showed that IPC reduced fluid requirements, lung oedema, lactate, TNF-α production and a more than fivefold increase of intestinal haem oxygenase-1 (HO-1) expression (Tamion *et al.*, 2002).

1.5.2 Antioxidants

The human body is endowed with many natural antioxidants which unfortunately are not capable of protection against the attack of oxidant formation during IR injury. It has been shown in many animal studies that supplementation of these anti-oxidants is helpful in reducing IR induced tissue damage (Table 1.2). The agents that have been used are allopurinol (Ahmadinejad *et al.*, 1996), superoxide dismutase (SOD)

(Cuzzocrea *et al.*, 2001), iron chelator (e.g.desferrioxamine) (Balogh *et al.*, 2002) ,N-acetyl cysteine (NAC) (Cuzzocrea *et al.*, 2000), ethanol (Yamaguchi *et al.*, 2002), ascorbic acid (Nakamura *et al.*, 1997), tocopherol (Gunel *et al.*, 1998), pentoxyfylline (Sener *et al.*, 2001;Hammerman *et al.*, 1999), captopril (Buyukgebiz *et al.*, 1994)and verapamil (Mocan *et al.*, 1995).

Intravenous SOD mimetic (M40401), when administered 15 minutes prior to reperfusion in a model of IRI preserved the architecture of the ileum, reduced the expression of cytokines and abolished the expression of P-selectin and ICAM-1 (Cuzzocrea *et al.*, 2001). In some cases two anti-oxidants are administered simultaneously (e.g. SOD and catalase) in an attempt to block the oxidants at more than one stage of formation (Simpson *et al.*, 1993). Kojima *et al.* showed that an intra-duodenal infusion of antioxidants glutathione, rebamipide and dymethylsulfoxide attenuated the increase in apoptosis in the intestinal mucosa after IR injury (Kojima *et al.*, 2003). A newly developed free radical scavenger, edaravone has shown to be effective in reducing IR injury of the intestine through its anti-cytokine and anti-chemokine effects (Tomatsuri *et al.*, 2004).

Table 1.2. A list of antioxidants used against reperfusion injury in the intestine

Agent	Author	Species	Mechanism	Outcome
Allopurinol	Ferrer(Ferrer *et al.*, 1998)	Rats	oxidative stress, scavenging free radicals	↓tissue damage, ↓Amylase, ↓MDA, ↓GSH & ↓MPO
Anti-thrombin III	Ozden(Ozden *et al.*, 1999)	Rats	lipid peroxidation	↓ MDA,↓ MPO,↓tissue dam
Bilirubin	Hammerman(Hammerman *et al.*, 2002)	Rats	lipid peroxidation	↓blood and intestinal thiobarbituric acid & tissue damage
	Ceran(Ceran *et al.*, 2001)	Rats	anti-oxidant	↓tissue damage
Edaravone	Tomatsuri(Tomatsuri *et al.*, 2004)	Rats	anti- cytokine & chemokine	↓tissue damage,↓MPO,↓TBA reactive substances, ↓CINC-protein & ↓CINC-1 mRNA
Captopril	Buyukgebiz(Buyukgebiz *et al.*, 1994)	Rats	free radical scavenger	↓ MDA,↓ MPO,↓tissue dam
Cyclosporin	Puglisi (Puglisi *et al.*, 1996)	Rats	free radical scavenger	↓tissue damage
Desferrioxamine	Balogh (Balogh *et al.*, 2002)	Rats	lipid peroxidation	↓MDA
Epidermal	Berlanga	Rats	lipid	↓MDA, ↓ MPO, ↓tissue

growth factor (EGF)	(Berlanga *et al.*, 2002)	Rabbits	peroxidation	damage
Glutamine	Harward (Harward *et al.*, 1994)	Rats	lipid peroxidation	↑GSH,↓conjugated diene (a by-product of lipid peroxidation)
Melatonin	Ustundag(Ustundag *et al.*, 2000)	Rats	oxidative stress	↑SOD, ↑glutathione peroxid
	Kazez (Kazez *et al.*, 2000)	Rats	lipid peroxidation	↓MDA &↓tissue damage
N-acetyl cysteine	Sun(Sun *et al.*, 2002)	Rats	neutrophil infiltration	↓ MPO & ↓endothelial permeability
Nitroglycerine	Dun(Dun *et al.*, 2001)	Rats	Lipid peroxidation	↓tissue damage, ↓MDA &↓LDH
Pyruvate	Cicalese(Cicalese *et al.*, 1996)	Rats	free radical scavenger	↓tissue damage,↓ free radica neutrophil infiltration
Rapamycin	Puglisi(Puglisi *et al.*, 1996)	Rats	free radical scavenger	↓tissue damage
SOD	Riaz(Riaz *et al.*, 2002b)	Rats	oxidative stress	↓neutrophil rolling & adhesi
Trimetazidine	Tetik(Tetik *et al.*, 1999)	Rats	free radical scavenger	↓MDA, ↓ MPO↓tissue dama
Verapamil	Mocan(Mocan *et al.*, 1995)	Rats	oxidative stress	↓tissue damage

CINC, cytokine-induced neutrophil chemoattractant; GSH, glutathione; HO-1, heme oxygenase-1; iNOS ,inducible nitric oxide synthase; LDH, lactate dehydrogenase; MDA , malonyldialdehyde; MPO, myloperoxidase; NO, Nitric Oxide; RNA, ribonucleic acid; SOD, superoxide dismutase; TBA*, thiobarbituric acid.

*TBA-an indicator of lipid peroxidation

1.5.3 NO Supplementation

Supplementation of NO has been shown to attenuate IR injury in intestine in cats and dogs (Kawata *et al.*, 2001;Aoki *et al.*, 1990).Kalia *et al.* showed that a spontaneous NO donor (FK-409) reduced both intestinal and lung damage after IRI of the intestine in rats (Kalia *et al.*, 2002). It was also shown that inhaled NO at low concentration exerts profound anti-leukocyte adherence effects in cat and dog IRI (Fox-Robichaud *et al.*, 1998). Whereas the use of selective inhibitors of iNOS have shown to be beneficial, the use of non-selective inhibitors have been shown to be deleterious in intestinal IR injury (Luo *et al.*, 2001).Recently, Naito *et al.* demonstrated that the administration of a selective iNOS inhibitor, ONO-1714 attenuated IRI of the intestine (Naito *et al.*, 2004). Intraluminal administration of nitroglycerine, a potent exogenous NO donor prevented the increase in intestinal permeability associated with IR injury of the intestine (Khanna *et al.*, 2003).

1.5.4 Anti-complement Therapy

IR injury is significantly reduced by complement inhibition, complement depletion or in complement deficient animals (Collard and Gelman, 2001) .Administration of a Complement (C5a) receptor antagonist has been shown to protect the intestine against the IR injury in a murine model (Heller *et al.*, 1999). Arumugam *et al.* showed that administration of an orally active C5a receptor antagonist was highly effective in reducing the damage to the intestine in a rat model (Arumugam *et al.*, 2002b). The anti-C5 antibody inhibited TNF-alpha expression and tissue injury. Zhao *et al.* showed that the use of a anti-murine C5 monoclonal antibody decreased neutrophilic infiltration of both the intestine and the lungs (Zhao *et al.*, 2002). Williams *et al.* showed that the administration of a complement inhibitor of soluble complement receptor 1 (sCR 1) attenuated intestinal mucosal injury, reduced the intestinal vascular hyperpermeabilty

and increased the survival in a rat model (Williams *et al.*, 1999). It has been demonstrated recently that the administration of human C1esterase inhibitor has been shown to attenuate reperfusion injury in a mouse model of intestinal IR injury (Karpel-Massler *et al.*, 2003).

1.5.5 Anti-leukocyte therapy

There are broadly three therapeutic strategies to attenuate leukocyte mediated IR injury. These are namely: (a) inhibition of leukocyte activation, (b) inhibition of leukocyte adhesion molecule synthesis and (c) inhibition of leukocyte-endothelial adhesion (Collard and Gelman, 2001).

The activation of leukocytes in IR injury is aided by the release of inflammatory mediators such as leukotriene-B_4, platelet activating factor (PAF), tumour necrosis factor-α (TNF- α) (Panes *et al.*, 1999). Studies have shown that the use of LT-B4 antagonists (Souza *et al.*, 2000b), PAF antagonists (Souza *et al.*, 2000a;Sun *et al.*, 2002) and the use of TNF-α inhibitors (Souza *et al.*, 2001) limits IR induced leukocyte activation.

A second strategy to attenuate leukocyte mediated IR injury has been to inhibit leukocyte adhesion molecule synthesis. Transcription factors regulating leukocyte adhesion molecule synthesis, such as NF-$_K$B are the targets for BAY11-7085 (Ikappa B inhibitor) (Zou *et al.*, 2003)and α melanocyte stimulating hormone (Hassoun *et al.*, 2002).

The third strategy is to limit the leukocyte-endothelial adhesion. Riaz *et al.* showed that the use of allopurinol and SOD reduced leukocyte rolling and adhesion in a dose dependent manner (Riaz *et al.*, 2002b). The use of monoclonal antibodies directed

against adhesion molecules reduces severity of IR injury in the intestine. One restraining factor that is limiting the clinical use of monoclonal antibodies against leukocyte-endothelial interaction is the possibility of an immune reaction (Harris and Granger, 1997). Pre-treatment with an anti P-selectin antibody have been shown to reduce leukocyte adhesion by about 85% and that IRI induced leukocyte adhesion was significantly reduced in leukocyte function antigen -1 (LFA) deficient mice (Riaz *et al.*, 2002a). However, Carmody *et al* demonstrated that eliminating P-selectin by using P-selectin knockout mice attenuated reperfusion injury and this beneficial effect was found to be independent of neutrophil adhesion (Carmody *et al.*, 2004). The administration of anti-LFA-1 antibodies have been shown to be effective in improving allograft survival in small bowel transplantation (Bowles *et al.*, 2000). A non exhaustive list of anti-leukocyte and anti-inflammatory agents used in IRI of the intestine is shown in Table 1.3.

Table 1.3 A list of anti-leukocyte and anti-inflammatory agents used against reperfusion injury in the intestine

Agent	Author	Species	Mechanism	Outcome
α-Melanocyte	Hassoun(Hassoun *et al.*, 2002)	Rats	Inhibition of NF-$_K$B	↓tissue damage & ↓MPO
Anti-IL-8 antibody	Tsuruma(Tsuruma *et al.*, 1996a)	Rats	Inhibition of IL-8 expression	↓tissue damage & TNF-α
Anti-TNF antibody	Koksoy(Koksoy *et al.*, 2001)	Rats	Inhibition of TNF expression	↓neutrophil sequestration
BAY-11-7095	Zou(Zou *et al.*, 2003)	Rats	Inhibition of I-kappa B	↓tissue damage > 30 mins of reperfusion & ↑tissue damage 6hrs > reperfusion,↓MPO,↓IL-6 & ↓ICAM-1
BN 52021	Droy-Lefaix(Droy-Lefaix *et al.*, 1991)	Rats	PAF antagonist	↓tissue damage
CP-105,696	Souza(Souza *et al.*, 2002)	Rats	Inhibition of leukotrienes	↓tissue damage, ↓vascular permeability & ↓TNF-α in the lungs & intestine
CINC Ab	Yagihashi(Yagih	Rats	Inhibition of TNF	↓tissue damage & ↓TNF-α

	ashi *et al.*, 1998)		expression	
Cyclic peptide AcF (OpdChaWR)	Arumugam(Arumugam *et al.*, 2002b)	Rats	Complement antagonist	↓tissue damage, ↓TNF-α & inhibition of IR induced neutropenia
Ethanol	Yamaguchi(Yamaguchi *et al.*, 2002)	Mice	Inhibition of neutrophil infiltration	↓leukocyte rolling & adhesion
Foetal bovine serum	Chen(Chen *et al.*, 2002)	Rats	Inhibition of IL-8	↓IL-8
FK-409	Kalia(Kalia *et al.*, 2002)	Rats	NO donor	Maintains WCC, ↓villi-leukocyte adhesion,↓ lung damage & ↑survival
	Kawata(Kawata *et al.*, 2001)	Dogs	NO donor	↓tissue damage
FR-167653	Yamamoto(Yamamoto *et al.*, 2001)	Rats	Inhibition of TNF-α & IL-1	↓tissue damage, ↓TNF,↓IL-1 & ↓AST
	Takeyoshi(Takeyoshi *et al.*, 1996)	Dogs	Inhibition of TNF-α & IL-1	↓tissue damage

GPI 6150	Mazzon(Mazzon *et al.*, 2002)	Rats	p-selectin & ICAM-1 blocker	Improves mean BP, ↓tissue damage, ↓MPO & ↑ survival
Glycine	Lee(Lee *et al.*, 2002)	Rats	Inhibition of neutrophil infiltration	↑mucosal protein & DNA,↓MPO
	Iijima(Iijima *et al.*, 1997)	Mice	Inhibition of IL-6	↓tissue damage, ↓bacterial translocation&↑ survival
Heparin binding EGF	Xia(Xia *et al.*, 2003)	Rats	Inhibition of neutrophil infiltration	↓expression of P/ E- selectins,VCAM-1 & ICAM-1
Hepatocyte growth factor	Kuenzler(Kuenzler *et al.*, 2002a)	Rats	Inhibition of TNF	↓caspases
Hyperbaric oxygen	Yang(Yang *et al.*, 2001)	Rats	Inhibition of TNF	↓TNF-α & lung MPO
IL-10	Lane(Lane *et al.*, 1997)	Mice	Cytokine attenuation	↓tissue damage, ↓TNF-α ,↓lung MPO & ↓IL-6
Inosine	Dowdall(Dowdall *et al.*, 2002)	Rats	Cytokine attenuation	↓tissue damage,↓ gut permeability & ↓lung MPO

L-Propionyl Carnitine	Stroh(Stroh *et al.*, 1998)	Rats	Inhibition of neutrophil infiltration	↑survival, maintains mean arterial pressure, ↓tissue MPO
Lipocortin-1	Cuzzocrea(Cuzzocrea *et al.*, 1997)	Rats	Inhibition of neutrophil infiltration	Maintains blood pressure, ↓tissue MPO & ↓tissue damage
L-Arginine	Warnecke(Warnecke *et al.*, 2002)	Rats	Inhibition of TNF-α & IL-6	↓tissue damage,↓TNF-α,↓hyaluronic acid & ↓IL-6
Lexipafant	Sun(Sun *et al.*, 2002)	Rats	Platelet activating factor antagonist	↓endothelial permeability & ↓MPO
M40401	Cuzzocrea(Cuzzocrea *et al.*, 2001)	Rats	p-selectin blocker & inhibition of ICAM-1	Improved blood pressure, ↓MDA & ↓MPO
Magnolol	Loong(Loong *et al.*, 2001)	Rats	Inhibition of IL-6	↓tissue damage &↓IL-6
Methyl Prednisolone	Warnecke(Warnecke *et al.*, 2002)	Rats	Inhibition of TNF & IL-6	↓tissue damage,↓TNF-α,↓hyaluronic acid & ↓IL-6
P-selectin glycoprotein ligand	Farmer(Farmer *et al.*, 2002)	Rats	p-selectin blocker	↓tissue damage, ↓IL-11,IL-6 ,interferons & ↓MPO

Pentoxifylline	Sener(Sener *et al.*, 2001)	Rats	Inhibition of neutrophil infiltration	↓MDA,↓MPO & ↑glutathione
Pirfenidone	Arumugam(Arumugam *et al.*, 2002a)	Rats	Inhibition of TNF-α	↓tissue damage, & ↓TNF-α
Rolipram	Souza(Souza *et al.*, 2001)	Rats	Inhibition of TNF-α	↓intestinal & lung permeability, ↓recruitment of neutrophils,↓IL-10,↓TNF-α in both lungs & intestine
UK 74505	Souza(Souza *et al.*, 2000a)	Rats	Platelet activating factor antagonist	↓TNF-α, ↓MPO, ↓tissue damage & mucosal permeability

Ab, antibody; AST, aspartate aminotransferase; BP, blood pressure; CINC, cytokine-induced neutrophil chemoattractant; DNA, deoxyribonucleic acid; EGF, epidermal growth factor; GSH, glutathione;HO-1, haem oxygenase-1;ICAM , intercellular adhesion molecule; IL, interleukin; iNOS ,inducible nitric oxide synthase ; LDH, lactate dehydrogenase; MDA , malonyldialdehyde; MPO, myloperoxidase; NF-$_{K}$B, nuclear factor kappa -B; NO, Nitric Oxide; SOD, superoxide dismutase; TNF, tumour necrosis factor; VCAM, vascular cellular adhesion molecule; WCC, white cell count.

1.5.6 Perfluorocarbons (PFCs)

Perfluorocarbons are hydrocarbon molecules in which hydrogen atoms are replaced by fluorine. They can dissolve 20 -25 times more oxygen than plasma at room temperature. The release of oxygen is facilitated by a low oxygen-binding constant causing a linear relationship between oxygen saturation and partial pressure. This results in release of oxygen to the tissues. Several studies have been shown that PFCs attenuate IR injury of the intestine (Floyd *et al.*, 1987;Oldham *et al.*, 1987;Ricci *et al.*, 1985).

Intraluminal administration of oxygenated PFCs into the intestine has been shown to preserve mucosal function and integrity after IR injuryof the intestine (O'Donnell *et al.*, 1997). Ohara *et al.* showed that peritoneal lavage with PFCs reduced the intestinal and pulmonary damage following IR injury of the intestine (Ohara *et al.*, 2001).Whereas Vejchapipat *et al.* demonstrated that an extraluminal administration of PFCs did not protect the intestine from IR injury in a rat model (Vejchapipat *et al.*, 2002). The small intestine when placed in a bag containing PFCs attenuated the expression of cytokines and decreased the intestinal damage in a rat model of IR injury (Fujino *et al.*, 2003a). Partial liquid ventilation with PFCs have been shown to reduced remote organ injury by reducing the lung damage (Shashikant *et al.*, 2002).

1.5.7 Enteral feeding

Enteral nutrition has been shown to reduce the death rate and remote organ injury in a mouse model (Fukatsu *et al.*, 2001). Enteral feeding maintains the mucosal immunity and the resistance to infections. Fukatsu *et al.* showed that five days of gut "starvation" with parenteral nutrition increases ICAM-1 and P-selectin expression, thereby attracting the PMNs to the intestine compared with enterally fed animals (Fukatsu *et al.*, 2000).These ICAM-1 changes were reversible with reinstitution of enteral feeding. Five days of re-feeding the parenterally fed mice with chow returned ICAM-1 levels to normal. Further more, lack of

enteral feeding has shown to alter the levels of IL-4 and IL-10 in the lamina propria (Wu *et al.*, 1999). IL-4 and IL-10 inhibit endothelial expression of ICAM-1.

1.5.8 Glutamine Supplementation

Glutamine considered to be a non-essential amino acid, appears to be a conditionally essential nutrient during serious injury (Lacey and Wilmore, 1990).Glutamine is the most abundant amino acid in the plasma and skeletal muscle under normal physiological conditions, but levels fall substantially after injury or surgery (Askanazi *et al.*, 1980). Several studies demonstrate that L-glutamine and glutamine dipeptide (glycyl-glutamine and alanyl-glutamine) supplemented parenteral nutrition and L-glutamine-supplemented enteral feeds decrease bacterial translocation, diminish experimental colitis and enhance mucosal adaptation with refeeding or following bowel resection (Wilmore, 1999;Ziegler *et al.*, 1999). In a porcine model of segmental small bowel autotransplantation, Li *et al.* showed glycyl-glutamine enriched parenteral nutrition significantly increased graft mucosal glutamine and protein levels and decreased intestinal permeability and bacterial translocation (Li *et al.*, 1999). Of interest, in a rat small bowel transplantation model, significantly improved jejunal graft maltose absorption and post-transplantation mortality were demonstrated when harvested grafts were cold-preserved in 1% L-glutamine versus saline alone or in University of Wisconsin preservation solutions (Sakawaki *et al.*, 1998).

Intravenous infusion of L-glutamine has been demonstrated to increase the cytoprotective HO-1 and the antioxidants GSH and SOD, with concomitant decrease of TNF-α in IR injury of the intestine (Tamaki *et al.*, 1999). Glutamine-enriched tube feeding was shown by Zhang et al to enhance transplanted small bowel growth, nutrient absorption and mucosal barrier function (Zhang *et al.*, 1995). Ikeda *et al.* in a mouse model of IR injury of the intestine showed that supplementation of TPN with glutamine partially supports mucosal immunity by

preserving respiratory and intestinal IgA levels, maintaining the proper IgA-stimulating cytokine milieu within the intestine, reducing intestinal ICAM-1 expression and neutrophil adhesion (Ikeda *et al.*, 2002). Wischmeyer provided evidence that glutamine enhances heat shock protein (HSP) expression and thereby confers protection against a variety of stressful stimuli (Wischmeyer, 2002;Kelly and Wischmeyer, 2003). Another probable mechanism is that glutamine given intravenously increased mucosal glutathione (GSH) levels and attenuates IR injury induced cell membrane lipid peroxidation (Harward *et al.*, 1994).

1.5.9 Glycine Supplementation

L-Glycine, a non-essential amino acid protects the intestine from IRI (Lee *et al.*, 2001;Lee *et al.*, 2002;Iijima *et al.*, 1997). Glycine has an impressive catalogue of functions such as anti-inflammatory, immunomodulatory and direct cytoprotective functions (Lee *et al.*, 2001;Lee *et al.*, 2002;Grotz *et al.*, 2001). Iijima *et al.* showed that the administration of 20% glycine attenuated mucosal damage, preserved the intestinal protein content, reduced bacterial translocation and improved the overall survival in a murine model (Iijima *et al.*, 1997). Glycine acts on inflammatory cells such as macrophages to suppress the activation of transcription factors and the formation of free radicals and inflammatory cytokines (Kelly *et al.*, 2003). Recently, Jacob *et al.* demonstrated that supplementation of glycine caused a downregulation of pro-apoptotic bax and caspase-3, whereas bcl-2 was upregulated (Jacob *et al.*, 2003).

1.6 Conclusions

IR injury of the intestine is a complex, multi-factorial pathophysiological process that involves the actions of oxygen derived free radicals, cytokines, nitric oxide and PMNs. Despite the complexity, attenuation of the reperfusion injury through ischemic and pharmacological preconditioning has been successfully applied to animal models of intestinal

IR injury. IPC has been found to be the most promising strategy against IR injury during the last few years, appearing to increase the tolerance of the intestine to reperfusion injury. However, although IPC has been shown beneficial in the human heart and the liver, prospectively controlled studies in humans involving IPC of the intestine is lacking. Research focussed on the application of novel drugs that can mimic the effects of IPC to manipulate the cellular events during IR injury of the intestine is obligated.

Chapter 2 Materials and Methods

2.1 Animals

2.1.1 Animal preparation and surgical procedure

The study was conducted under a project license granted by the home office in accordance with the Animals (Scientific Procedures) Act 1986. Male Sprague- Dawley rats, each weighing 250- 300g were used for the experiments. All animals were kept in temperature controlled environment with 12 hours light- dark cycle and allowed tap water and standard rat chow pellets ad libitum. Animal research protocols were approved by the hospital ethics committee.

Animals were anaesthetised using isoflurane (Baxter, Norfolk, UK) and allowed to breathe spontaneously via concentric mask connected to an oxygen regulator during the procedure. The animal's body temperature was maintained at 36-38 $^{\circ}$C using a heating pad (Harvard apparatus Ltd., Kent, UK) and monitored with a rectal temperature probe. The Arterial oxygen saturation (SaO_2) and heart rate (HR) were continuously monitored with a pulse oximeter (Ohmeda® Biox 3740 pulse oximeter, Ohmeda, Louisville, Colorado, USA). The left carotid artery was cannulated with a polyethylene catheter (0.76-mm inner diameter, Portex, Kent, UK) and connected to a pressure transducer for monitoring of mean arterial blood pressure (MABP). The right jugular vein was cannulated with a smaller polyethylene catheter (0.40-mm inner diameter, Portex, Kent, UK) for administering normal saline (1 ml/100g body weight/hr) to compensate for intra-operative fluid evaporation. All animals had a bolus of heparin (20U/kg, intravenously) to prevent potential thrombus formation in the ischaemic segment of the intestine due to haemostasis.

Laparotomy was carried out through a midline incision. The superior mesenteric artery (SMA) was identified and dissected out from the mesentery to enable the passage of a 4/0 vicryl™ (Ethicon, Edinburgh, UK) suture loop. The SMA was occluded according to the method described by Arumugam *et al*(Arumugam *et al.*, 2002b). The free ends of the vicryl suture were then passed through a 5 cm segment of polyethylene tube (1.4- mm inner diameter). The tube was then gently advanced over the suture onto the mesentery and fixed in place with a haemostat. Reperfusion started when the haemostat was released. The animal's abdomen was covered with a plastic wrap (Saran wrap) to prevent fluid evaporation. At the end of the experiment the animals were killed by exsanguination.

2.1.2 Blood and tissue sampling

At the end of the reperfusion period, blood samples (1 ml each) were collected from the inferior vena cava for the mesurements of plasma Lactate dehydrogenase, alanine transminase, aspartate transminase. Samples were heparinised and centrifuged at 2,000 g for 10 minutes at room temperature to sediment the erythrocytes. The plasma supernatent was removed and stored at –80 °C until required for assay.

Sections of ileum were (1) freeze clamped in liquid nitrogen for haem oxygenase determination and immediately stored at –80 °C for western blotting of HO-1, and (2) fixed in 10% formalin for histological study. Biopsies of the lung tissue were obtained at the end of the reperfusion were fixed in 10% formalin for histological analysis.

2.2. Assessment of intestinal microvascular perfusion by laser Doppler flowmetry

2.2.1 Introduction

The laser Doppler flowmetry (LDF) which was first used in 1972 by Riva *et al.* to measure the retinal microcirculation in rabbits (Riva *et al.*, 1972) has been used widely in the study of a variety of transparent tissues such as the web of frog (Koyama *et al.*, 1975), mesentery of the mouse (Born *et al.*, 1978), hamster cheek pouch (Einav *et al.*, 1975), human skin (Holloway, Jr. and Watkins, 1977) and testicular blood flow(Damber *et al.*, 1982). This technique has been subsequently used in the study of various organs perfusion such as the pancreas(Hosotani *et al.*, 1985), the kidneys (Hansell and Ulfendahl, 1986), intestine (Shepherd and Riedel, 1982) and the liver (Koti *et al.*, 2002b).

2.2.2 Principles

The measurement of blood flow with the aid of LDF is based on the detection of a shift in the frequency of the backscattered light after the moving red blood cells (RBCs) are hit by an incident collimated laser beam according to the Doppler principle. When a beam of laser comes into contact with the tissue, the backscattered light is composed of two portions, the first consists of the portion from static tissue matrix, which has not been Doppler shifted, and the second portion is composed of a broad spectrum of frequencies from interaction with the moving RBC's. Therefore, the Doppler signal varies linearly with the product of the total number of moving RBCs in the measured volume of a few cubic millimeters multiplied by the mean velocity of these RBCs. The LDF measurements are expressed in arbitrary perfusion units (flux). The application and reproducibility of LDF measurement for assessment of intestinal microcirculation has been validated in both experimental animals (Ahn *et al.*, 1985) and human small bowel transplantation (Corbett *et al.*, 2000).

2.2.3 The equipment

A commercial laser Doppler flowmeter (LDF) was used. The basic structure of a LDF consists of a probe, optic fibres and the instrument. The probe transmits laser light to tissue via optical fibre and back scattered light return from tissue to the flowmeter, then to two separate photo-detectors in the instrument.

2.2.4 Calibration of Laser Doppler flowmeter

The intestinal microvascular blood flow in this study was assessed using a commercially available dual channel surface LDF (DRT4, Moor Instruments Ltd., Axminster, Devon, UK) (Figure 2.1). The LDF was calibrated before each study against a standard reference (Brownian motion of polystyrene microspheres in water) provided by the manufacturer.

2.2.5 Recording of the intestinal microvascular blood flow

The LDF technique has been widely validated to estimate perfusion in the gastrointestinal tract (Ahn *et al.*, 1985;Corbett *et al.*, 2000;Feld *et al.*, 1982;Shepherd and Riedel, 1982;Thollander *et al.*, 1997). To minimise any disturbance to blood flow by the LDF probe pressure on the tissue, the probe was mounted on a probe holder so that the actual probe was just in contact with the intestinal surface without any pressure applied by the weight of probe. The probe was placed to a fixed site on the intestine to avoid any error due to anatomical variation in the microcirculation.

Data from the continuous measurement by LDF was collected at the relevant points in each experiment and calculated as a mean of 1-minute data.

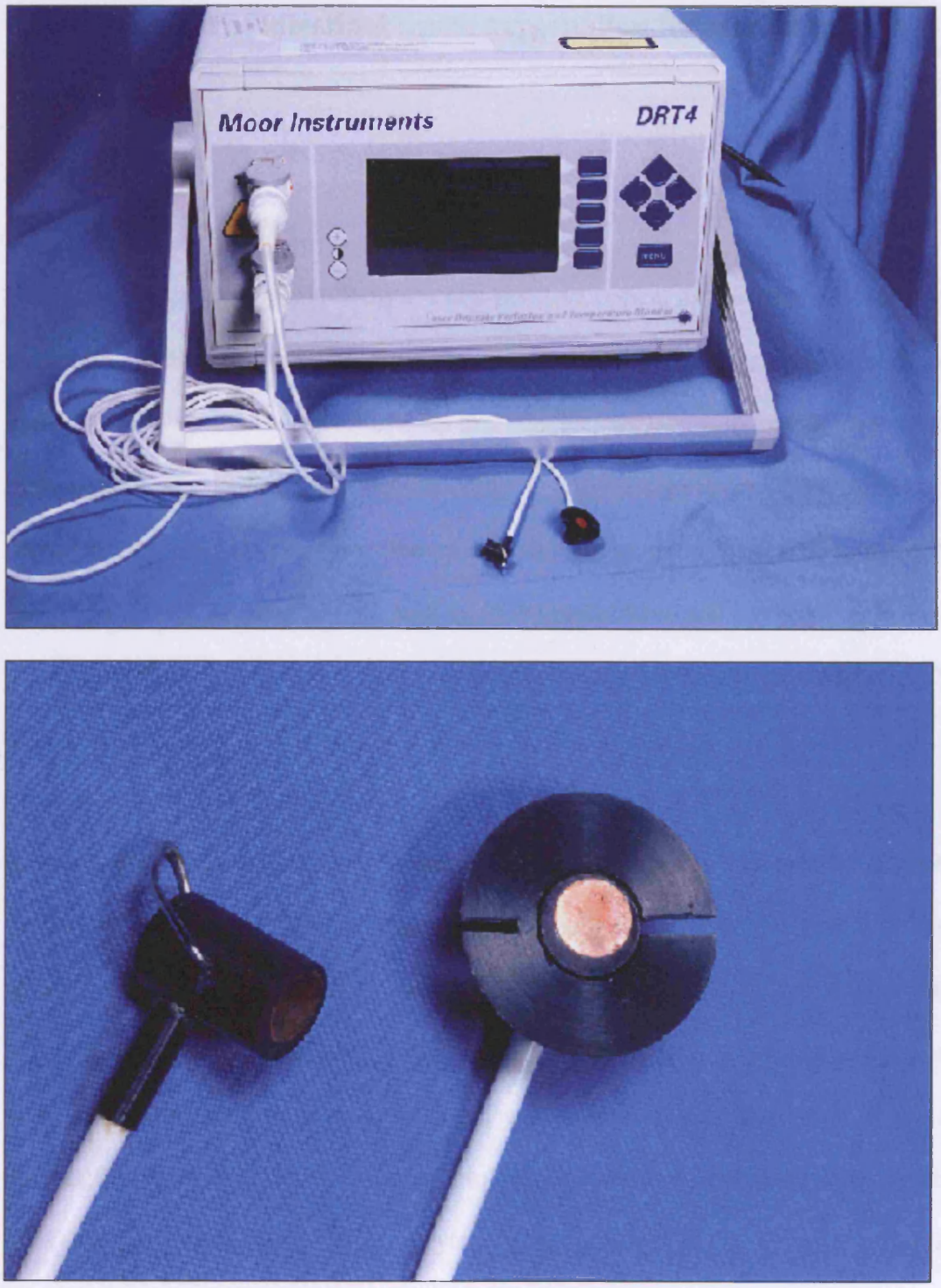


Figure 2.1. Dual channel surface laser Doppler flowmeter and its probes

(DRT4, Moor Instruments Ltd., Axminster, UK).

2.3. Assessment of intestinal tissue oxygenation by near infrared spectroscopy

2.3.1 Principles

Near infrared spectroscopy (NIRS) is a non-invasive optical method that can assess oxygen availability and consumption within a biologically living tissue. The evaluation of oxygen delivery to tissues is crucial as it assures that adequate oxygen is available for oxidative phosphorylation to proceed at a rate matching with the energy needs of the cell. Oxidative phosphorylation requires an intact mitochondrial electron transport system wherein NADH and $FADH_2$ from the tricarboxylic acid cycle are re-oxidized and oxygen is reduced by four electrons to form water, accompanied by generation of adenosine tri-phosphate (ATP) (Fig 2.2).

In vivo application of NIRS was first described by Jobsis in 1977(Jobsis, 1977). NIRS utilizes the ability of NIR light (700-1000 nm) to pass through liver, intestine , brain with much less scattering than occurs with shorter wavelengths of light. The interactions of light within the tissue involves the combination of reflectance, scattering and absorption which depends upon many factors including the light wavelength and the illuminated tissue type (Jobsis, 1977). Although in the NIR region of the spectrum the absorption of light by oxyhaemoglobin (HbO_2), deoxyhaemoglobin (Hb) and cytochrome oxidase (Cyt Ox) decreases significantly, compared to that observed in the visible region of the light, their absorption spectra remain different allowing spectroscopic separation of the two compounds using only a few wavelengths(Cope *et al.*, 1988;Wray *et al.*, 1988).

The technique of NIRS relies upon two main physical properties: (1) the relative transparency of biological tissue to light in the NIR region of the spectrum and (2) the existence of different

tissue chromophores with characteristic absorption spectra in the NIR light spectrum (Elwell *et al.*, 1994;Wray *et al.*, 1988)(Fukatsu *et al.*, 1999). In tissue with homogeneous scattering the calculation of light attenuation and the relationship between the optical absorption and chromophore concentration may be described by a modified Beer - Lambert's law. The law modifications include (a) an additive term, G, due to scattering losses and (b) a multiplier, to account for the increased optical pathlength due to scattering. This law can be used to convert the obtained optical densities to concentration changes of Hb, HbO_2, and Cyt Ox in μmole/L per optical pathlength (Cope *et al.*, 1988;Wray *et al.*, 1988;Fukatsu *et al.*, 1999): $A = \alpha . c . d . B + G$.

where A is the attenuation of light (optical density), α is the absorption coefficient of the chromophore ($\mu mole^{-1}.cm^{-1}$), c is the concentration of the absorbing compound (μmole/L) and d is the geometrical distance between the points where light enters and leaves the tissue (cm). B the differential pathlength factor (DPF) which accounts for the increase in optical pathlength due to light scattering (which causes the optical pathlength to be greater than d) and G is a constant geometrical factor which accounts for loss of photons by scattering. As G cannot be quantified in vivo and is dependent upon the scattering coefficient of the tissue interrogated, it is not possible to measure the absolute concentration of the chromophore in the tissue from measurement of the absolute attenuation. If α, B, and d are known and G assumed to remain constant during measurement, we can measure the change in the chromophore concentration (Δc) from measuring the change in attenuation (ΔA) from the following formula: $\Delta c = \Delta A / \alpha . d . B$.

Since the absolute concentration of tissue chromophores are unknown and cannot be calculated due to the effect of light scattering within the tissue, all NIRS measurements are expressed as absolute concentration changes (μmole/L) from an arbitrary zero at the start of the measurement.

The absorption coefficients of HbO_2 and Hb can be obtained in cuvette studies on lysed human blood, for the oxygenated spectra these cuvettes are bubbled with different oxygen saturation (Wray *et al.*, 1988)(Fukatsu *et al.*, 1999). The absorption coefficient of Cyt Ox was obtained in vivo from the brains of experimental animals whose blood had been replaced by a blood substitute (fluorocarbon) with exposure to 100% O_2 or N_2 to obtain the oxidised and reduced Cyt Ox spectra (De Blasi *et al.*, 1997)(Fukatsu *et al.*, 1999).

B is dependent upon the amount of scattering in the medium which can be measured by 'the time of flight' method (Delpy *et al.*, 1988).

For simultaneous computation of the changes in concentration of a number of chromophores from changes in attenuation at a number of wavelength, a mathematical operation (algorithm) can be used which incorporates the relevant absorption coefficient for each chromophore at each wavelength (Fukatsu *et al.*, 1999).(Delpy *et al.*, 1988)As there are three chromophores of interest in the tissue (HbO_2, Hb, and Cyt Ox) it is necessary to make measurements at a minimum of three wavelengths and if more than three wavelengths are used, standard curve-fitting analysis may be used to increase the accuracy of the calculated concentration changes (Cope *et al.*, 1988;Wray *et al.*, 1988)(Delpy *et al.*, 1988).

2.3.2 Equipment

In its simplest form, the equipment required for a NIRS system includes the following:

(a) Light sources that can generate multiple wavelengths of NIR light. In order to elucidate information on HbO_2, Hb and Cyt Ox, four wavelengths are generally required.

(b) Optical bundles (optodes) to transmit the photons from the source to the monitoring site and from the monitored site to a detector.

(c) Photon detection circuitry (e.g., a photomultiplier and integration amplifier).

(d) A computer with appropriate software (algorithms) to process the information obtained in the recovered light and compare it with the known amount of light delived.

(e) An information display system.

The NIR spectrometer used in this study is the NIRO 500 (Hamamatsu Photonics K.K., Hamamatsu, Japan) (Figure 2.3 and 2/4). This spectrometer is the commercial version of an instrument developed by colleagues in the Department of Medical Physics and Bioengineering, University College London(Cope *et al.*, 1988). In the NIRO 500, the light source is monochromatic light generated from semiconductor laser diodes (LD). The light is produced at four wavelengths (774, 826, 849, and 906 nm).

The light is produced by laser diodes and carried to the intestine via a bundle of optical fibres in sequential pulses. The optical fibres are covered by a light proof protective sheath and its distal end terminated in a very small glass prism which reflects the light through 90^{o} to direct it into the tissue(Elwell *et al.*, 1994). Photons emerging from the intestine are collected by the second bundle of optical fibres and detected by a photomultiplier tube (PMT) light detector. The incident and transmitted light intensities are recorded and from these the changes in the concentration of tissue chromophores (μmole/L) are calculated using an algorithm incorporating the known chromophores absorption coefficients and an experimentally measured optical pathlength (see below).

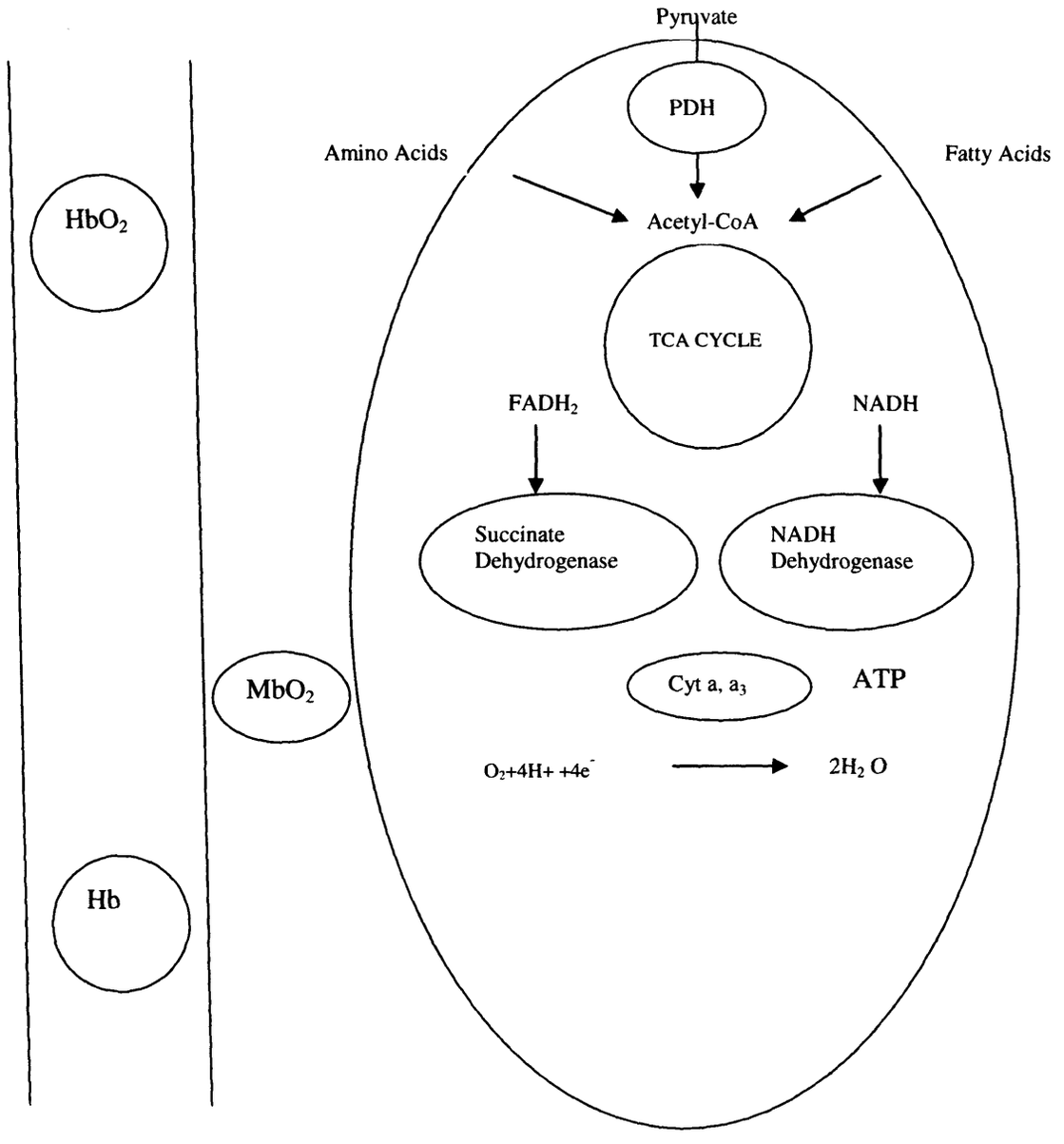


Figure 2.2. Schematic diagram depicting oxygen delivery in a cell.

Oxygen is delivered to the capillary bed by oxyhaemoglobin(HbO2) following which it diffuses into the cell and is bound to myoglobin (MbO2) and utilised by cytochrome oxidase (Cyt Ox), the terminal cytochrome in the electron-transport chain. Glycolysis generates pyruvate, which is transported into the mitochondria and oxidized by the tricarboxylic acid (TCA) cycle. Reducing equivalents in the form of FADH2 and NADH are produced to enter the electron-transport chain which generates ATP by oxidative phosphorylation. The redox state of Cyt Ox is determined by the flow of electrons through the electron-transport chain and by the availability of oxygen; thus reflecting the overall activity of oxidative metabolism in the cell.

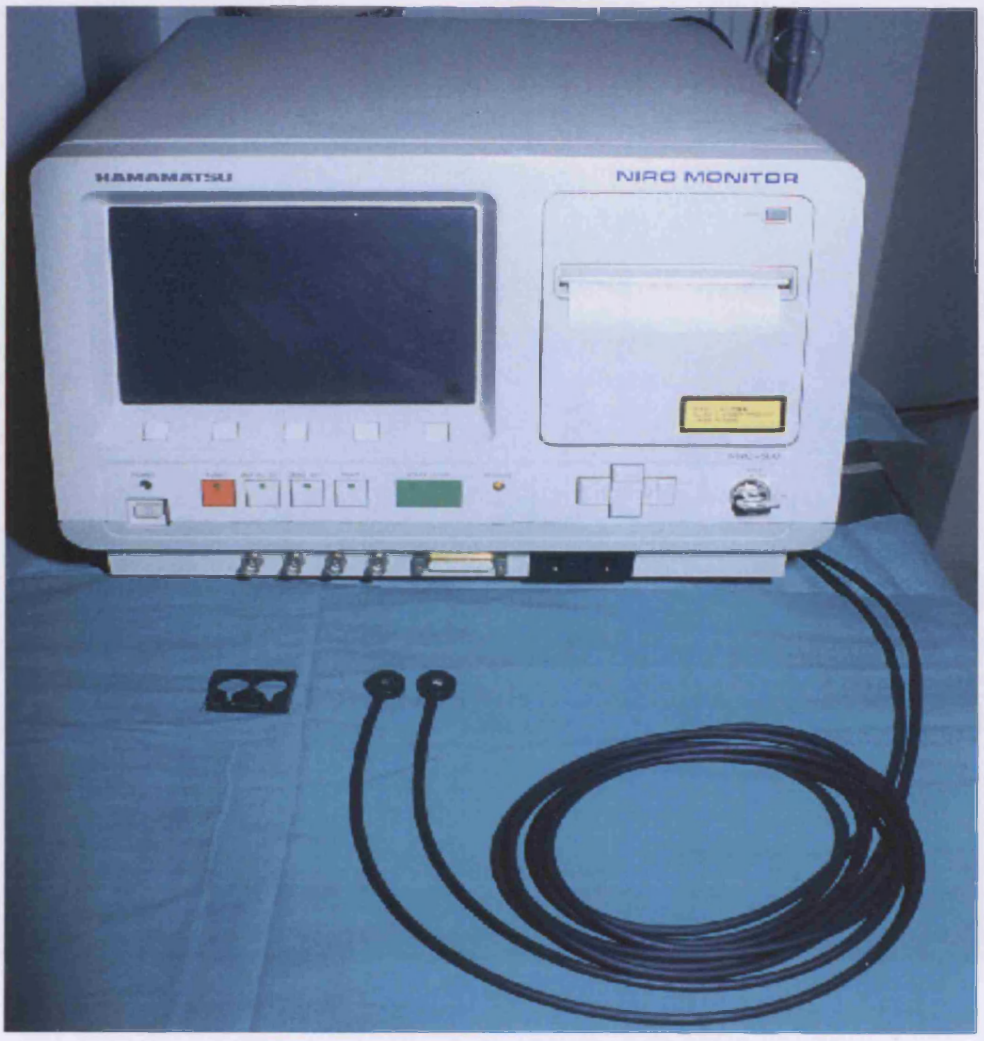


Figure 2.3. Near infrared spectrometer

(NIRO 500, Hamamatsu Photonics KK, Hamamatsu, Japan).

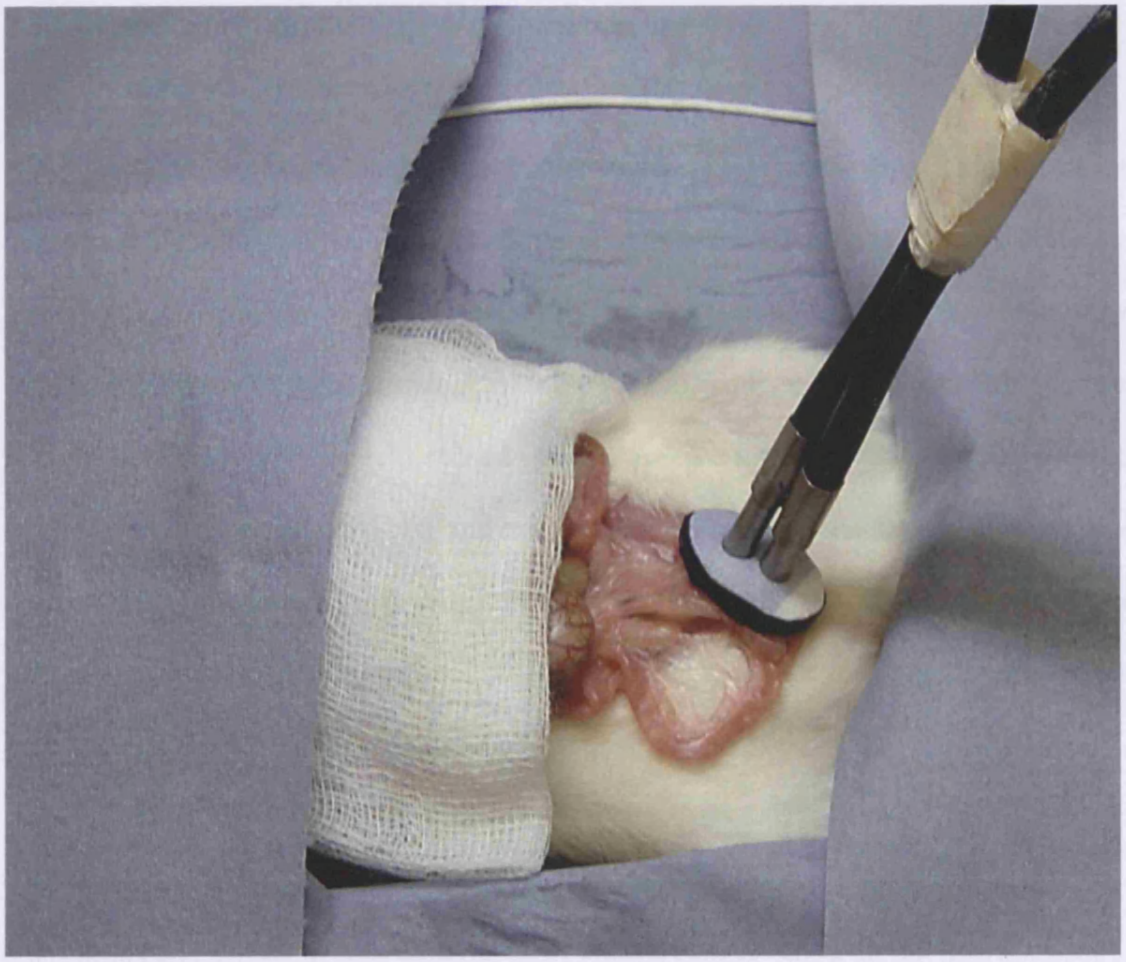


Figure 2.4. NIRO 500 probes and a rubber optode holder.

2.3.3 Recording of intestinal tissue oxygenation in the rat

NIRS probes were mounted inside a probe holder and placed on the serosal surface of the intestine at a fixed site in the ileum in all the animals in each experiment to avoid any anatomical variation which could influence in tissue oxygenation and blood volume. As a part of the modification of this instrument for use on the intestine, a flexible rubber holder was made to hold the NIRS probes at a fixed spacing over the intestine surface. This probe holder ensured that the sites of light entry and exit are maintained at a constant and known spacing distance which minimises the possibility of artefact due to changes in the distance between the probe ends. Also, it allows a satisfactory contact between the intestine surface and the probe ends (Figure 2.4).

The NIRO includes the facility to set the attenuation and therefore chromophore concentration) changes to zero with the NIRO initial setting. Since all the measurements are changes from an arbitrary initial zero, this function in important to ensure that artefacts such as system drift, optode movement, and excessive light having a minimal effect on the data. For collection of NIRS data, a sampling time of 1 second was used. The NIRS data were continuously collected in a laptop computer connected to the NIRS. These data are the changes in light attenuation (optical densities: OD) at four wavelengths due to absorption by the tissue chromophores. A software program called ONMAIN® (Hamamatsu Photonics K.K., Hamamatsu, Japan) was used to convert these data into changes in concentration changes of HbO_2, Hb, and Cyt Ox (μmole/L) using the previously defined algorithm in the NIRO 500. This was then transferred to Excel® data sheets (Microsoft Company, Seattle, USA) for analysis. The data at the relevant time points were collected as the mean of 1-minute data and calculated in regard to the baseline value at the start of the experiment.

2.3.4 Limitations

Three practical factors affect the results obtained using NIRS monitoring. First, uninterpretable NIRS signals may occur due to movement or motion artefact. Second trend monitoring in the same area over a period of time requires maintaining a constant optode placement and geometry. Finally when a deep tissue is the area of interest for monitoring, the contribution of overlying superficial tissues needs to be considered. This is affected most by the chromphore content of the superficial tissues and by optode geometry, specifically, the separation distance of the incident light source and the detector.

Optode separation is an important determinant of the area of interrogation by the incident photons. In general, photons follow an elliptical path through tissue from the source to the detector(Chance *et al.*, 1992). The shorter the interoptode distance, the less deep is the penetration of light and a smaller area of tissue studied. On the other hand, an extreme separation of emitter and detector results in increased photon scatter and more absorption of photons by tissue chromophores, resulting in a weak signal that may be difficult to interpret.

2.4 Assessment of portal venous blood flow by ultrasound transonic flowmeter

2.4.1 Principles

In 1966, the first ultrasound flowmeter was made by Franklin and co-workers, which worked on the ultrasound transit-time method. Unlike the Doppler principle that measures the frequency shift, the reflected sound from one moving target makes possible the measurement of the time differences over a given distance between the upstream and downstream sound waves. Similar to LDF, the first ultrasound flowmeter depended on velocity measurement of a

point over an area of flow and required an assessment of the inner diameter of the vessel to determine the flow volume (Transonic-Systems 1993).

2.4.2 The ultrasound flowmeter

The ultrasound flowmeter used in the subsequent studies is shown in Fig 2.5.

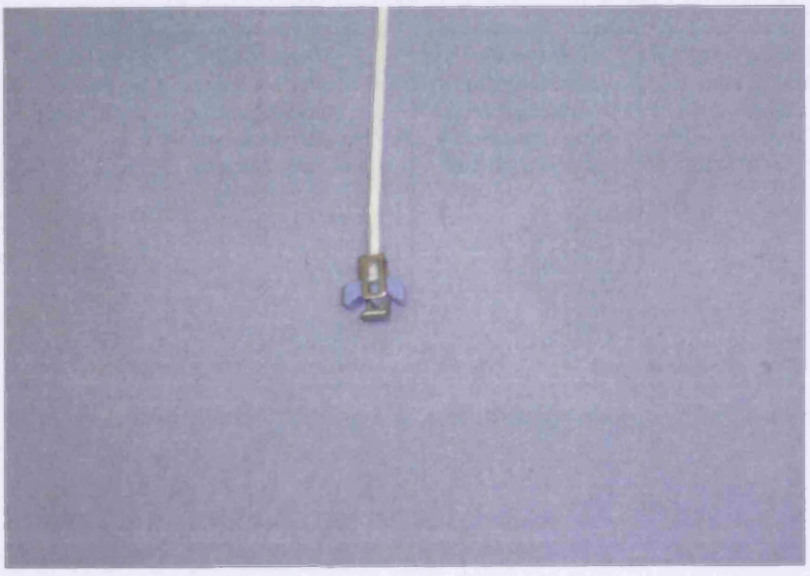
The probe of the mentioned ultrasound flowmeter consists of two adjacent sensor windows and a metallic acoustic reflector between which lies on the blood vessel. The upstream transducer first sends out sound impulses that passe through the blood vessel and the surrounding tissue. The impulse then reaches the downstream transducer where the sound impulse is converted into a starting signal which is then amplified and sent to a phase detector. The sound velocity is elevated as it travels downstream, resulting in shortening of transit time of the arriving impulse. The starting signal and the reference signal from a rhythm generator from a phase difference that is recorded and saved.

The next cycle is just an exchange in function between the sound wave sender and signal converter. The sound wave travelling from the downstream transducer, which now acts as the sound wave sender, to the upstream transducer (i.e. signal converter). Upstream travelling causes a delay in transit time. The resulting phase shift is then subtracted from the first one to give the flow volume independent of the measurements of the blood vessel. The electronic detector is so sensitive that the time difference in measuring a flow rate up to 25 ml/min is as small as one picosecond.

Many flowmeters require a certain angle between the incident direction of the probe and the blood vessel (i.e. the vector of flow). Such a measurement is sensitive to changes in the probe

direction and disturbances in laminar flows, for example, secondary flows and wavy flow patterns arising from the bending course of the blood vessel. The problem is solved by the design of the Transonic flowmeter probe which offers a double capturing of a reflected sound wave. The vector sum of the two measurements corresponds to the axial flow components. This technique makes the flow measurement independent of the flow profile and the incident angle of the probe (Transonic-systems 1993). As flow sensitivity is optimised for a specific range of vessel diameters, accurate blood flow measurement for blood vessel of different size requires different probes (Transonic-systems 1997).

Figure 2.5. A ultrasound transonic flowmeter



Ultrasonic probe

2.5 Assessment of intestinal microcirculation by intravital fluorescent microscopy

2.5.1. Introduction

The first intravital fluorescent microscope (IVFM) was made by Ellinger and Hirt in Germany , which was a modified version of the fluorescence microscope (developed in the early 20th century) so that it could be used to examine opaque tissues(Kasten, 1993).

The IVFM can be divided into two separate measuring systems. The first represents the microscope alone, with the light source, lenses, filters and fluorescence acting as the functional elements (Fig 2.6).

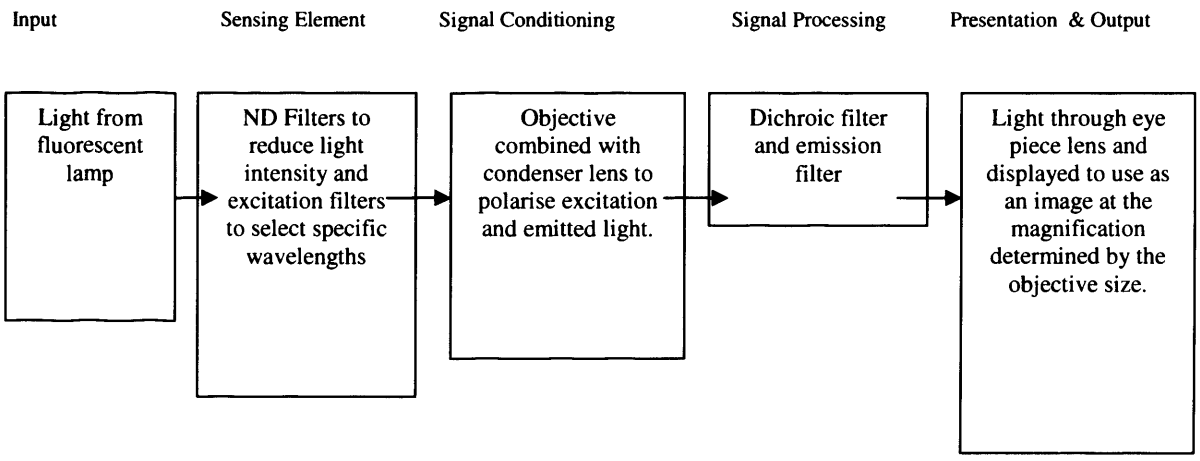


Figure 2.6. Intravital fluorescence microscope – instrumental components.

The second system begins with the image seen from the microscope being converted via the CCD camera, passing through the frame grabber and onto the computer monitor display (Figure 2.7)

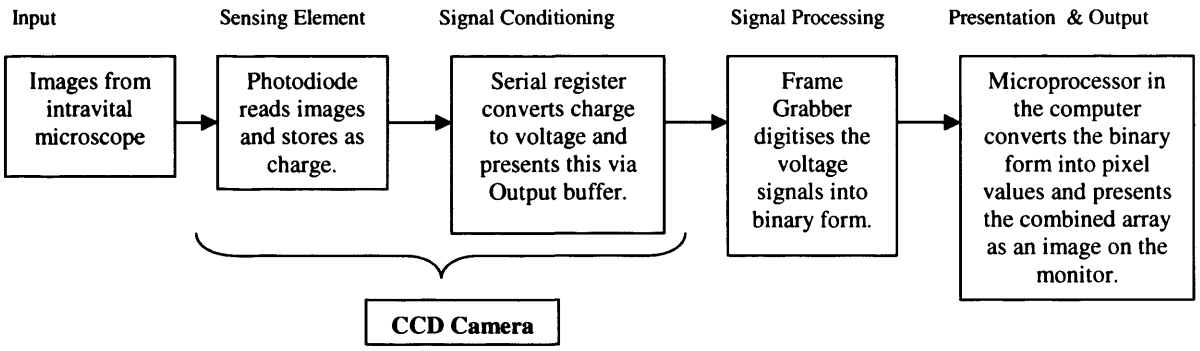


Figure 2.7.5 Image recording system in the intravital microscope

The IVFM used for this study was a custom built Nikon Epi-illumination system with filter block set suitable for Texas Red, FITC and DAPI dyes as detailed in table 2.1. (Fig 2.8)

Table 2.1. The details of filter set for the Nikon Epi-illumination system.

Fluorochrome	**Excitation (nm)**	**Emission (nm)**	**Colour**	**Type of label**
DAPI	360	450	Blue	Nuclear Stain
FITC	495	525	Green	Protein Conjugation
Texas Red	596	620	Red	Protein Conjugation

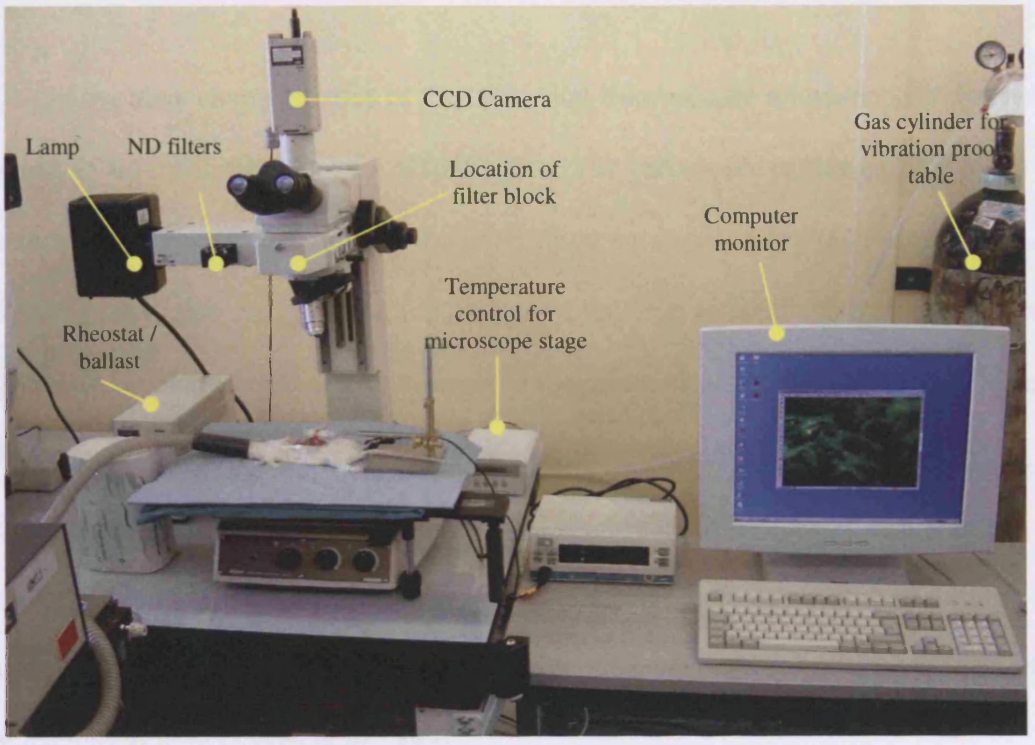


Figure 6.8.The complete setup of intravital microscopy.

2.5.2 Principles

One of the main characteristics of the intra-vital fluorescence microscope is that it works in aid of fluorescence for visualisation. The chromophores are excited by an external light source; they absorb the energy and pass into an excited energy state. After entering the higher energy state, the molecules undergo internal changes. The electrons in certain molecules instead of returning to the ground state enter a metastable state. When the molecules pass down to the ground state they emit the excess energy as electromagnetic radiation (Sykes *et al.*, 1991) – seen as fluorescence. The energy between the metastable and ground states is less than the energy absorbed during excitation, so the emission wavelength will be of longer wavelength than the absorbed or excitation light (Sykes *et al.*, 1991).

The light from the epi-illumination light source (which is usually a mercury arc or xenon lamp in standard IVFM setups) first passes through an excitation or short-pass (SP) filter allowing only the excitation waves through. These are then passed onto a chromatic beam splitter (dichromatic mirror) where wavelengths below a certain value are reflected onto the specimen while any above the wavelength value are passed through and dissipated. The light hitting the specimen then activates the fluorescence probe which produces emission light of a particular wavelength.
A dichromatic mirror allows passage of excitation wavelengths of certain values and emission wavelengths of certain values. According to Stokes' law the wavelength of emission is of lower energy and therefore longer wavelength than that of the excitation. The dichromatic mirror is designed to allow the transmission of longer *emission* wavelengths and reflect the shorter *excitation* wavelengths. The three filters are usually contained within a filter box as illustrated in figure 2.9.

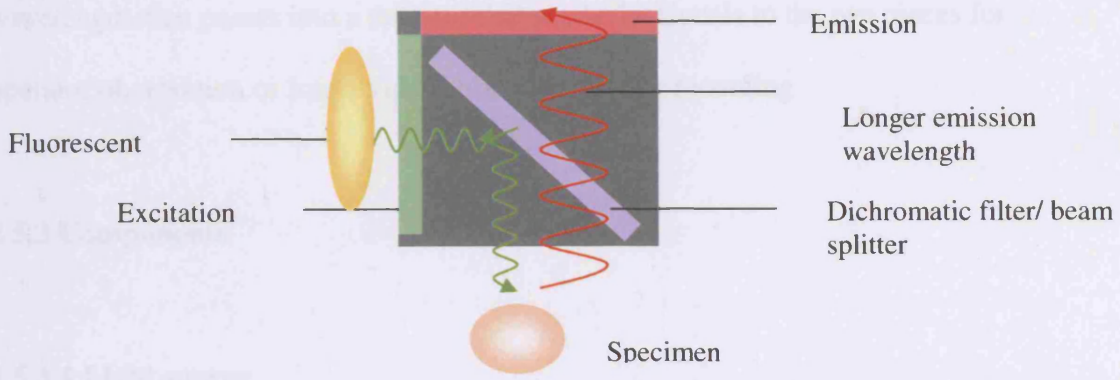


Figure 2.9. Schema of Filter Block

After passing through the dichromatic beam splitter the excitation wavelength then passes through a barrier filter that removes any light of shorter wavelength that may have mistakenly passed through the dichromatic beam splitter. This emission wavelength then passes into a detector that sends the signals to the eye pieces for operator observation or into a video camera for image recording.

2.5.3 Components

2.5.3.1 Light source

The light of the IVFM is a fluorescent mercury arc lamp, which is protected in a lamp-house. Since the development of epi-illumination, microscope optics has developed with the incorporation of laser light sources increasing the resolution of the image and improving the signal-to-noise ratio. A rheostat or ballast alters the current applied to the lamp to control the intensity of the light produced. In a gas discharge, such as a fluorescent lamp, current causes resistance to decrease. This is because as more electrons and ions flow through a particular area, they bump into more atoms, which frees up electrons, creating more charged particles.

2.5.3.2 Objectives

IVFMs usually use water immersion objectives as these reduce the refractive index in living in vivo imaging .The water immersion objective delivers contrast and resolution values nearly equivalent to the theoretical limits, and maintains its performance when water layers of 80 and 153 micrometers are added between the target specimen and

cover slip, a simulation of the situation encountered in imaging deep within aqueous material such as living cells or tissue.

There are four types of filters in the intra-vital fluorescence microscope system, neutral density (ND) filters, an excitation filter, an emission filter and a multi-band pass filter.

Special neutral density (ND) filters are built in the light path to block 90-95% of the incidence light passing into the microscope to prevent damage to the eye as well as reduce overexposure of fluorescence in specimens (Nikon, 2004). There are three neutral density filters which are placed in front of the optical light path to reduce illumination without altering the colour balance.

The excitation filter passes only a selected range of wavelengths of light to cause the fluorescently labelled specimen to fluoresce and filters the rest. The bandwidth of a filter determines the brightness of the fluorescent image. If the bandwidth is narrow then the image appears dark but minimal auto-fluorescence and photo-bleaching occur. With a wide bandwidth, although the image appears bright, autofluorescence may also be detected with the added disadvantage of photo-bleaching (Nikon, 2004).

The emission or barrier filter allows only light wavelengths that have been emitted from the specimen. These are usually longer according to Stoke's law. In older epi-illumination microscopes only a dichromatic beam splitter is present allowing use of one fluorescent dye at a time. However newer developed models have a multi-band pass filter or a polychromatic beam splitter that can allow visualisation of three different coloured fluorescent dyes.

2.5.3.3 CCD Camera

The image obtained from the IVFM is then recorded by a charged coupled device (CCD) camera. For this study a JVC TK-C1360B colour video camera was used. CCD sensors are light integrating devices that accumulate photo charges until image readout. The CCD chip in the camera contains an array of pixels that transform light (wavelength 400nm to 1000nm) into a charge, which during readout is transformed into a voltage.

Once photo charges are shifted to the storage area, images are erased from the CCD light sensing area (Cinelli, 1998). New photo charges can not pile up on top of the previous images. In video rate CCD cameras, this process occurs at regular video-rate of 60 Hz in RS 170 format. However the JVC camera used in this study produces an image acquisition rate of 50 Hz.

The photo charges are shifted in block from the sensing area to the storage area, then each line is individually read to the serial registers and finally photo charges are transferred to the output buffers. Each readout cycle is initiated by a vertical sync pulse (vertical blanking sync) which activates the parallel driver and triggers the shift block of photo charges accumulated in the sensing area to the CCD storage area.
Exposure time is the main factor that determines the sensitivity of CCD cameras. Long exposure times improve camera sensitivity and reduce the noise levels of the images, since the accumulation of the photo charge in the CCD sensor is proportional to the duration of the exposure period .The analogue video images are digitised at varying resolutions typically around 8 bits resolution by a frame grabber board.

2.5.3.4 Frame Grabber

The Matrix Meteor II/Standard frame grabber used in this study allows image acquisition at 25 frames per second. The main parts of the frame grabber are the low-pass filter, the decoder, the trigger and the image coding components. The low pass filter reduces the high frequency noise and aliasing effects from the analogue CCD signals and passes the refined signals to the video decoder. This is the component of the frame grabber that performs the actual analogue to digital conversion of the component (Y/C) analogue video signals.

2.5.3.5 Image Analysis Software

Adjusting the output image can enhance the resolution and contrast of the image. Light intensity and colour are represented in numbers between 0 (black) and 255 (white). Converting the image into binary form means it is converted into a black and white image. This is done by assigning threshold values which determine the distribution of the pixels into two populations either of value 0 or 255.

An image can be transformed into binary form using many different types of software packages. Laboratory Universal Computer Image Analysis (LUCIA) is a multi-spectral image analysis software developed specifically for image processing independently on red, green and blue components and then combining them together into the RGB image at the same time. Most Nikon microscopes are supplied with Lucia software and there are many versions available denoted by the letter following Lucia. Lucia G is the top of the range package that allows 24-bit colour image analysis with the function to allow user to create specialized macros.

2.6 Haem Oxygenase Assay

2.6.1 Activity assay

HO activity in ileal microsomal fractions was measured using a spectrophotometric assay of bilirubin production according to the method described previously (Motterlini *et al.*, 1996). Briefly, tissue microsomes were added to the following mixture: $MgCl_2$ (2 mM) phosphate-buffered saline (100 mM, pH 7.4; Sigma, UK), rat liver cytosol as a source of biliverdin reductase (3 mg total protein), hemin (10 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U) and NADPH (0.8 mM). The reaction was conducted in the dark for 30 min at 37°C and terminated by the addition of chloroform. All the above chemicals were purchased from Sigma, UK. The amount of extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm and an extinction coefficient of 40 $mM^{-1}cm^{-1}$ was used for bilirubin. The total protein content of the samples was determined using a colorimetric assay according to the manufacturer's instructions (Bio-Rad, UK) and bovine gamma globulin was used as a standard.

2.6.2. HO expression detcted by Western blotting

Haeme Oxygenase protein was identified using Western blotting. Fresh samples of ileal tissue (100- 200 mg) were homogenised in TOXEX buffer (20 mM HEPES [pH 7.9], 0.35 mol/L NaCl, 20% glycerole, 1% Nonidet P-40, 1 mmol/L $MgCl_2$, 0.5 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.1 mmol/L ethylene glycolbis(b-aminoethyl ether)-*N*,*N*-tetraacetic acid [EGTA], 100 mmol/L dithiothreitol [DTT], 0.1% phenylmethylsulfonyl fluoride [PMSF], 10 mg/mL aprotinin) on ice, incubated for 30 minutes, and centrifuged at 13,000 rpm for 5 minutes. For each lane, 100 mg of protein was dissolved in 10 mL of 13 sodium dodecyl sulfate loading dye and boiled for 5

minutes. A biotinylated protein marker (New England Biolabs, Schwalbach, Germany) was added. The samples were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA) by electroblotting for 2.5 hours (Semidry Trans-Blot, BioRad, Hercules, CA). The membrane was blocked in a buffer containing 20 mmol/L Tris-base (pH 7.6), 137 mmol/L NaCl, 3.8 mL 1 mol/L HCl/L, 0.1% Tween (13 TBST), and 5% low-fat dry milk powder for 1 hour and incubated with a rabbit polyclonal anti–HO-1 antibody (1:1,000 dilution; SPA 895, StressGen, Biotechnologies, Victoria, British Columbia, Canada) or a mouse monoclonal anti-HSP 70 antibody (1:1,000 dilution; SPA 810, StressGen, Biotechnologies) in 13 TBST and 5% low-fat dry milk for 2 hours at room temperature. After 3 washing steps with 1 X TBST, a secondary anti-rabbit or anti-mouse antibody (1:10,000 dilution; ECL-detection kit, Amersham Pharmacia, Freiburg, Germany) and horseradish peroxidase– conjugated anti-biotin antibody (1:1,000, New England Biolabs) was added and incubated for 1 hour in 1 X TBST and 5% low-fat dry milk. Following 2 washing steps with 1 X TBST and 2 washing steps with 1 X TBS, detection was performed by the ECL detection kit (Amersham Pharmacia) according to the manufacturer's instructions. After this the membrane was exposed to a digital camera as part of an electronic imaging system to visualise the proteins bound to the antibody.

2.7 Histological investigations

At the end of the experiment, samples of ileum and lungs were removed, fixed in 10 per cent neutral buffered formalin and embedded in paraffin; paraffin section 4μm thick were cut using a microtome and mounted on slides for haematoxylin and eosin staining.

Assessment of ileal injury was performed with light microscopy without knowledge of study groups by a scoring system devised by Chiu *et al* (table 2.2)(Chiu *et al.*, 1970).

Table 2.2. Scoring system for assessment of ileal injury.

Grade	Description
0	Normal villi
1	Subepithelial space top of villus
2	Extension of subepithelial space at top of villus with moderate separation of mucosa from lamina propria
3	Extensive subepithelial separation from the lamina propria down the sides of the villi, ulceration at the villous tips
4	Denuded villi
5	Disintegration of lamina propria

Chapter 3 Ischaemic preconditioning improves the microvascular perfusion and oxygenation following reperfusion injury of the intestine

3.1 Introduction

The effect of ischaemic preconditioning (IPC) on the intestinal microvascular perfusion and oxygenation is poorly understood.

The intestine is one of the tissues that is extremely sensitive to ischaemia and it demands high blood flow to sustain oxygen consumption, accounting for approximately 20% of the body's total oxygen consumption (Bohlen, 1998). Tissue oxygenation reflects the adequacy of microvascular perfusion and is the ultimate determinant of viability. IR injury is associated with the breakdown of microvascular perfusion with subsequent impairment of tissue oxygenation (Turnage *et al.*, 1995). Therefore, changes in intestinal tissue oxygenation with IPC may give an important indication of the effect of IPC on IR injury.

Near infrared reflectance spectroscopy (NIRS) is one of the methods under development for assessing the adequacy of mesenteric tissue oxygenation. Varela *et al* demonstrated that NIRS was able to detect early changes in gastric tissue oxygenation, which highly correlated with parameters of mesenteric perfusion (Varela *et al.*, 2001). NIRS measures changes in haemoglobin oxygenation which gives useful information about extracellular tissue oxygenation(Koti *et al.*, 2002a). NIRS also measures the reduction oxidation (redox) changes of the copper center (CuA) of cytochrome oxidase (Cyt Ox)(El Desoky *et al.*, 2001) and therefore changes in the redox state of CuA in cytochrome oxidase can reflect changes in intracellular oxygenation and mitochondrial function(El Desoky *et al.*, 1999).

This study was designed to explore the effect of IPC, on the early phase of small bowel IR injury by focussing on the intestinal microvascular perfusion and oxygenation.

3.2 Materials and methods

Animal care and experimental protocols were performed as discussed previously in chapter 2.1.1.

3.3.1 Operative procedures

Animals were anaesthetised and operated upon as described previously in chapter 2.1.1.

3.3.2 Experimental protocol

Rats (n=18) were randomly allocated to one of 3 study groups (n=6/group).

Group I. Sham laparotomy, in which the SMA was identified and passage of vicryl suture was performed, but without vascular occlusion.

Group II. IR, in which SMA was occluded for 30 min, followed by a 2 hour period of reperfusion.

Group III. IPC+IR, the SMA was occluded for 10 min and released for 10 min. This was followed by IR (as in Group II).

3.3.3 Measurement of intestinal microvascular perfusion

Intestinal microvascular perfusion (IMP) was measured by a surface LDF (DRT4, Moor Instruments Limited, Axminster, UK) in flux units as described previously in chapter 2.2.5. LDF probe was placed on a fixed site on the serosa of the ileum and was held in place by a probe holder. Serosal blood flow has previously been shown to correlate well with mucosal flow (Corbett *et al.*, 2000). LDF data were collected continuously at sampling rate of 2Hz on computer.

3.3.4 Measurement of intestinal tissue oxygenation

Intestinal tissue oxygenation was measured using NIRS as described in detail previously in chapter 2.3.3. A NIRS adapted algorithm was used to measure continuously intestinal HbO_2, Hb, and Cyt Ox concentration changes in μmole/L. NIRS was optically initialised to zero at the start of the experiment. NIRS measurements after ischaemia or sham laparotomy were expressed relative to the baseline.

3.3.5 Measurement of portal venous blood flow

It has been reported that IR injury of the intestine is known to profoundly disturb the hepatic blood flow (Nakamura *et al.*, 2001), we studied the effect of IR injury and IPC on the portal venous blood flow (PVF). It was monitored continuously in ml/min, using a dual Transonic Medical Flowmeter system (HT207, Transonic Medical System Inc, New York, USA).(Fig 9). The transonic flowmeter perivascular probe (2 mm diameter) was placed around the portal vein (Yang *et al.*, 2003). This has been considered as a suitable technique for estimation of hepatic blood flow (Yang *et al.*, 2003).

3.3.6 Biochemical assays

Blood samples were taken from the carotid artery at the end of reperfusion. They were centrifuged at 2000 g for 10 min at room temperature to sediment the erythrocytes. The serum lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analysed on a Hitachi 747 auto-analyzer (Hitachi Ltd, Tokyo, Japan) by using commercially available enzymatic kits (Boehringer Mannheim Ltd., East Sussex, UK).

3.3.7 Histological investigation

At the end of the experiment, tissue samples of ileum were removed, fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections (4μm thick) were

cut using a microtome and mounted on slides for haematoxylin and eosin (H&E) staining. The histological slides were assessed under light microscopy without knowledge of study groups and ileal injury was scored by the method (table 1) described previously by Chiu *et al* (Chiu *et al.*, 1970).

3.3.8 Data collection and statistical analysis

Data from the pulse oximeter, pressure monitor, LDF, NIRS and the transonic ultrasonic system were collected continuously on laptop computer at rate of 2Hz. The data were calculated as 1-min averages at baseline, at the end of ischaemia and at 30, 60, 90 and 120 min of reperfusion. All values are expressed as mean ±standard error of the mean (SEM). Analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated, where unpaired Student's *t*-test was used for statistical analysis between groups. $p< 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Systemic haemodynamics parameters

There were no significant changes in HR and SaO_2 throughout the experiment in all three groups of animals (p=0.522). In the sham group the MABP did not change statistically throughout the experiment (p =0.153). During ischaemia there was a significant increase in MABP from the baseline in both IR (87±4 mmHg to 112± 5 mmHg, p=0.005) and IPC groups (88 ±7 mmHg to 112±5 mmHg, p=0.005). However, the MABP did not change statistically as compared with baseline at the end of the reperfusion period between all three groups (p =0.765) (Fig 3.1).

3.4.2 Intestinal microvascular perfusion

IMP did not alter significantly during the course of the experiment in the sham group ($p = 0.509$). There were significant differences between IR and IPC groups in IMP at the initial 30 min of reperfusion and this persisted till the end of the 2 hr reperfusion period (42.5±2.8 % in IR vs. 70.8±4.1% in IPC, $p<0.001$) (Fig 3.2). There was no significant difference in IMP at the end of reperfusion in IPC compared to the sham laparotomy group ($p=0.530$).

3.4.3 Intestinal tissue oxygenation

Intestinal tissue oxygenation did not alter significantly during the course of the experiment in the sham laparotomy group (Fig 3.3). In IR group, there was a significant decrease in HbO_2 and Cyt Ox with concomitant increase in Hb. During reperfusion, there was a significant decline in HbO_2 and Cyt Ox levels compared to the baseline.

IPC was associated with significant increased levels of HbO_2 and Cyt Ox ($p<0.001$ IPC vs. IR). The levels of HbO_2 increased significantly on reperfusion and this increase persisted throughout the reperfusion period. The levels of Hb on reperfusion showed an increase from ischaemic levels, however, it was statistically lower compared to the IR group.

3.4.4 Portal venous blood flow

PVF was reduced significantly during the period of ischaemia in both IR (17±3 % of baseline) and IPC (11±5% of baseline). At the end of the 2 hr reperfusion period IPC improved the PVF significantly (42±9% vs. 79±9%; IR vs. IPC, $p=0.005$). (Fig 3.4).

3.4.5 Biochemical analysis

At the end of reperfusion, the LDH value in sham group was 364± 88.9μ/L. It increased significantly in IR group (1973.8± 306.5μ/L) as compared with sham (P<0.001). However, LDH vallue was significantly lower (p< 0.001) in IPC (667.1±86.8μ/L) as compared to IR (Fig 3.5).

3.4.6 Histological analysis

IR resulted in a significant increase in the histological score as compared with sham group (3± 0.21.0± 0.2; IR vs. IR). IPC reduced the histological score significantly to 1.5± 0.2 (p =0.009) (Fig 3.6)

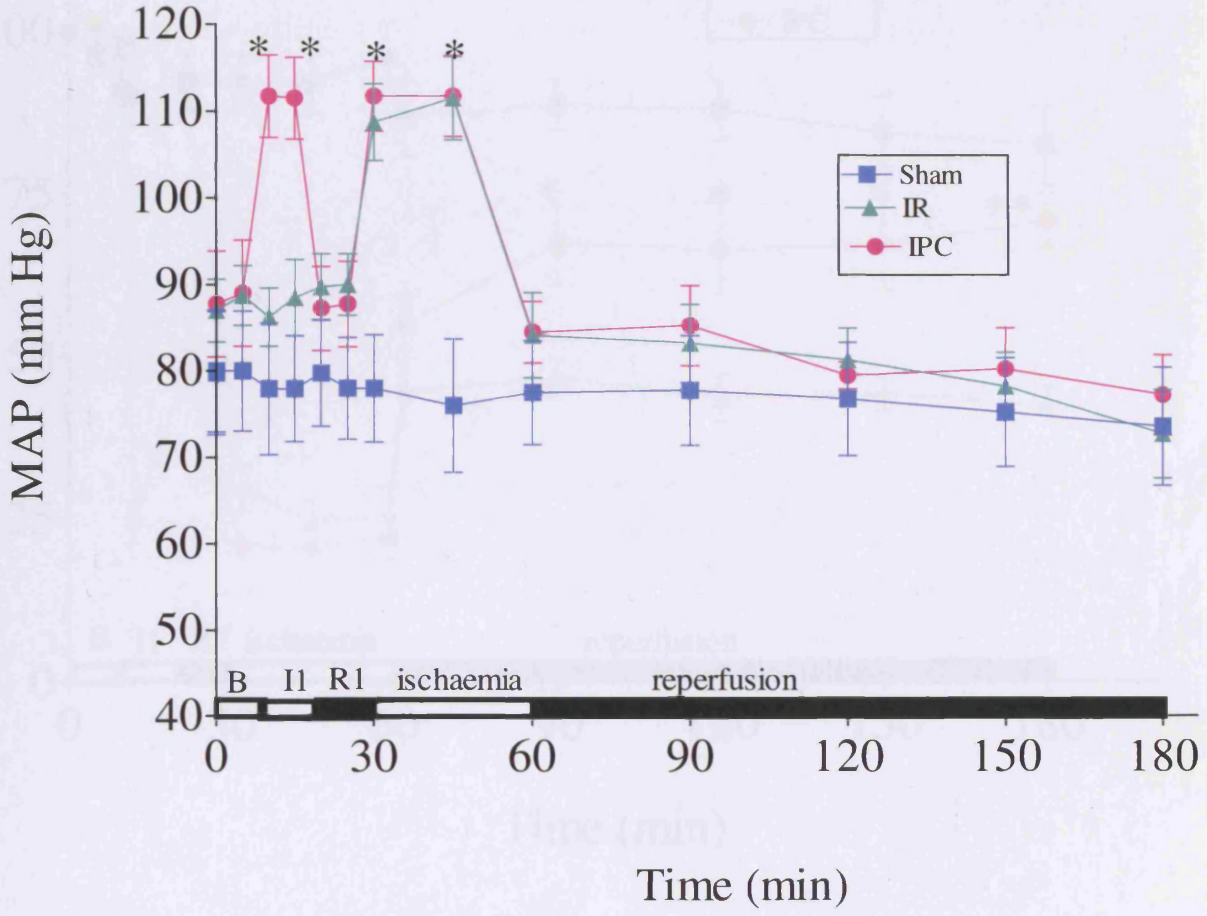


Figure 3.1. Mean arterial blood pressure measurements in all the three groups. Results are presented as mean± SEM of 6 animals from each group.

*p=0.005 vs. sham. B, baseline; I1, 10min of ischaemia; R1, 10 min of reperfusion.

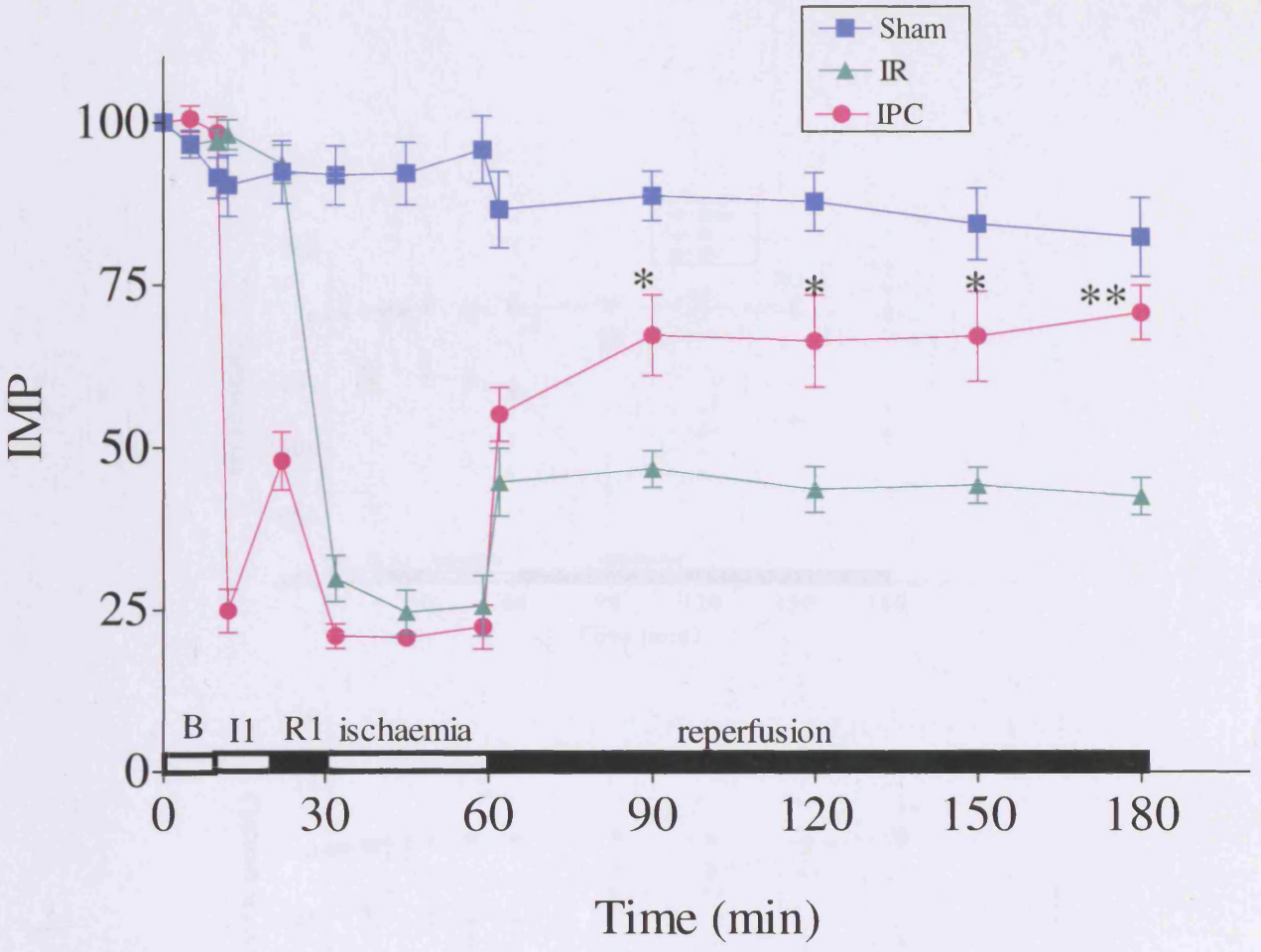


Figure 3.2. Intestinal microvascular perfusion (IMP) measured by LDF. For comparison, LDF readings were recorded as 100% at baseline. Values are mean ± SEM of 6 animals in each group.

* p=0.046 versus IR; ** p<0.001 vs. IR. B, baseline; I1, 10min of ischaemia; R1, 10 min of reperfusion

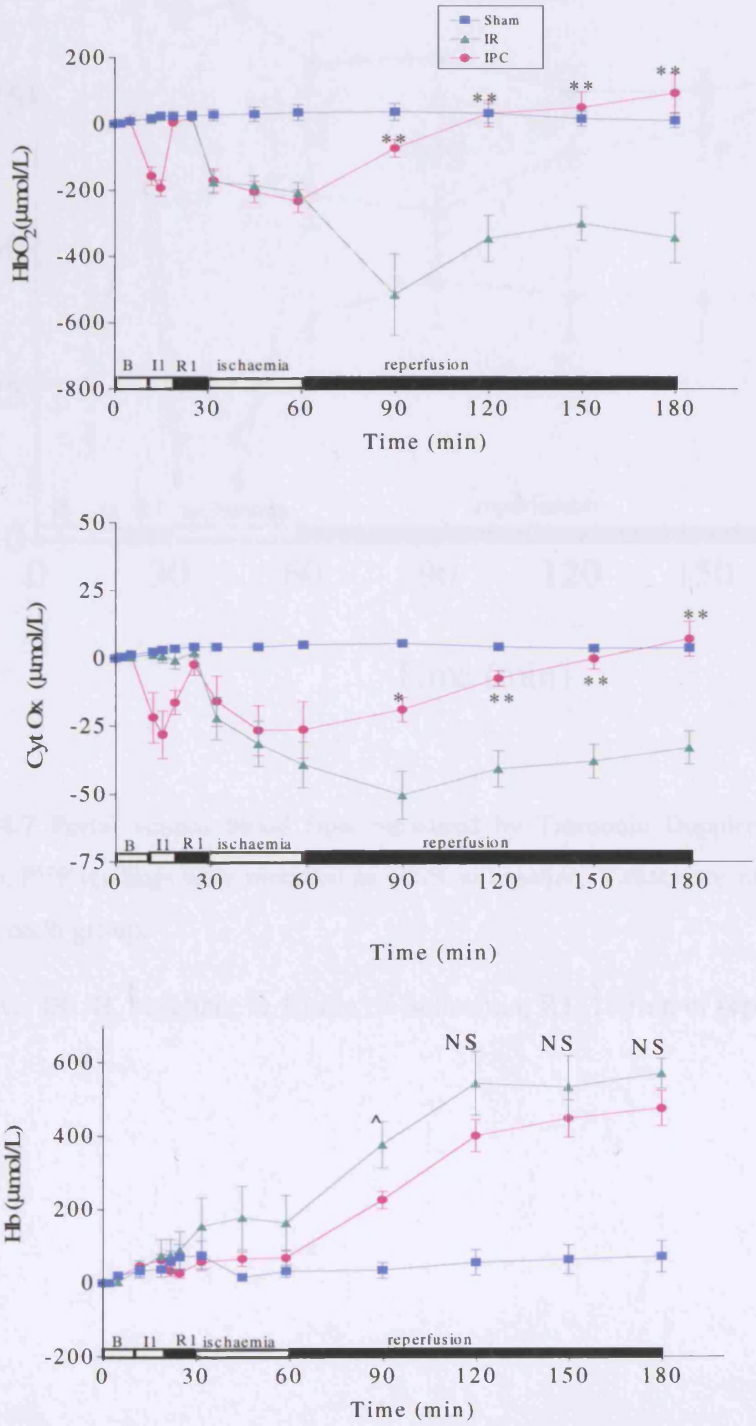


Figure 3.3. Intestinal tissue oxygenation measured by NIRS. *p= 0.045 IPC versus IR; ** p<0.001IPC versus IR; ^ p=0.038 IR vs. IPC; NS, non significant; B, baseline, I1, 10min of ischaemia; R1, 10 min of reperfusion.

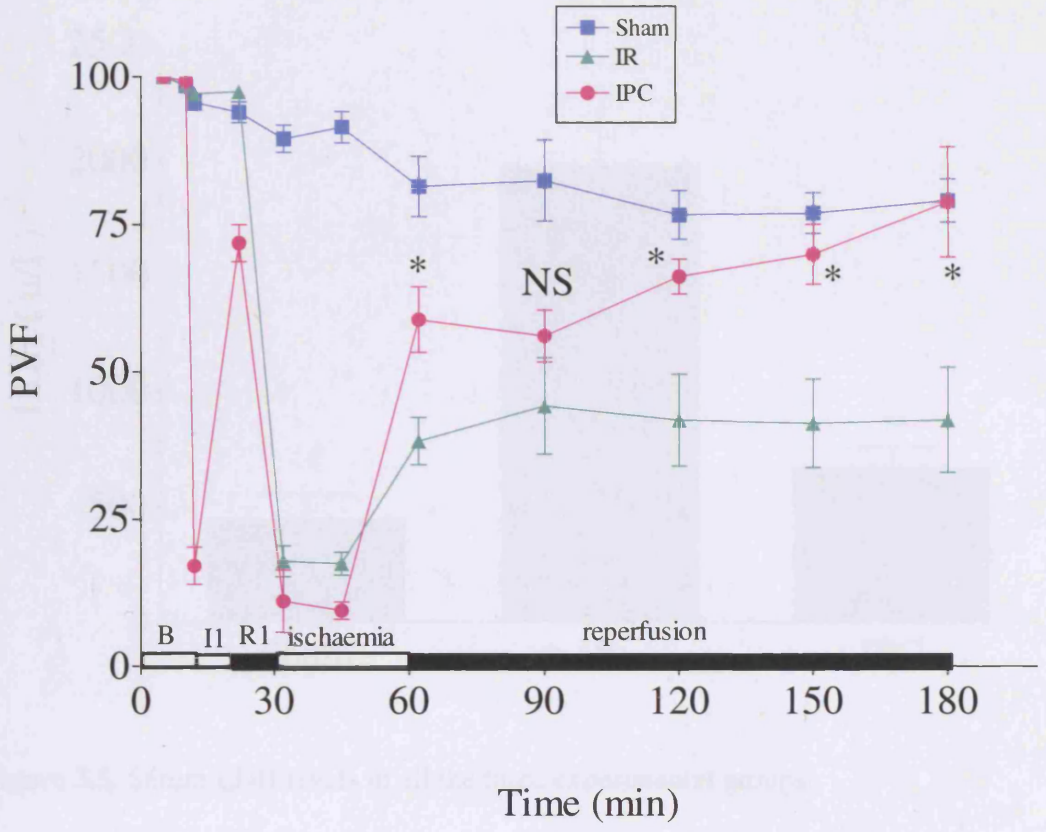


Figure 3.4.7 Portal venous blood flow measured by Transonic Doppler flow probe. For comparison, PVF readings were recorded as 100% at baseline. Values are mean ± SEM of 6 animals in each group.

*p=0.005 vs. IR. B, baseline; I1,10min of ischaemia; R1, 10 min of reperfusion.

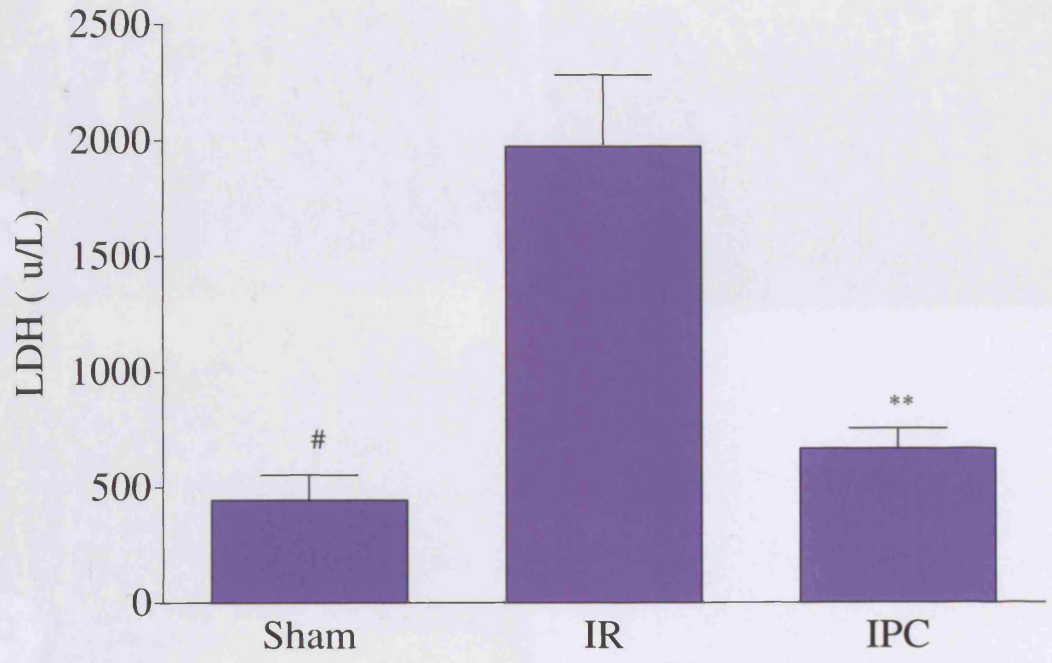


Figure 3.5. Serum LDH levels in all the three experimental groups.

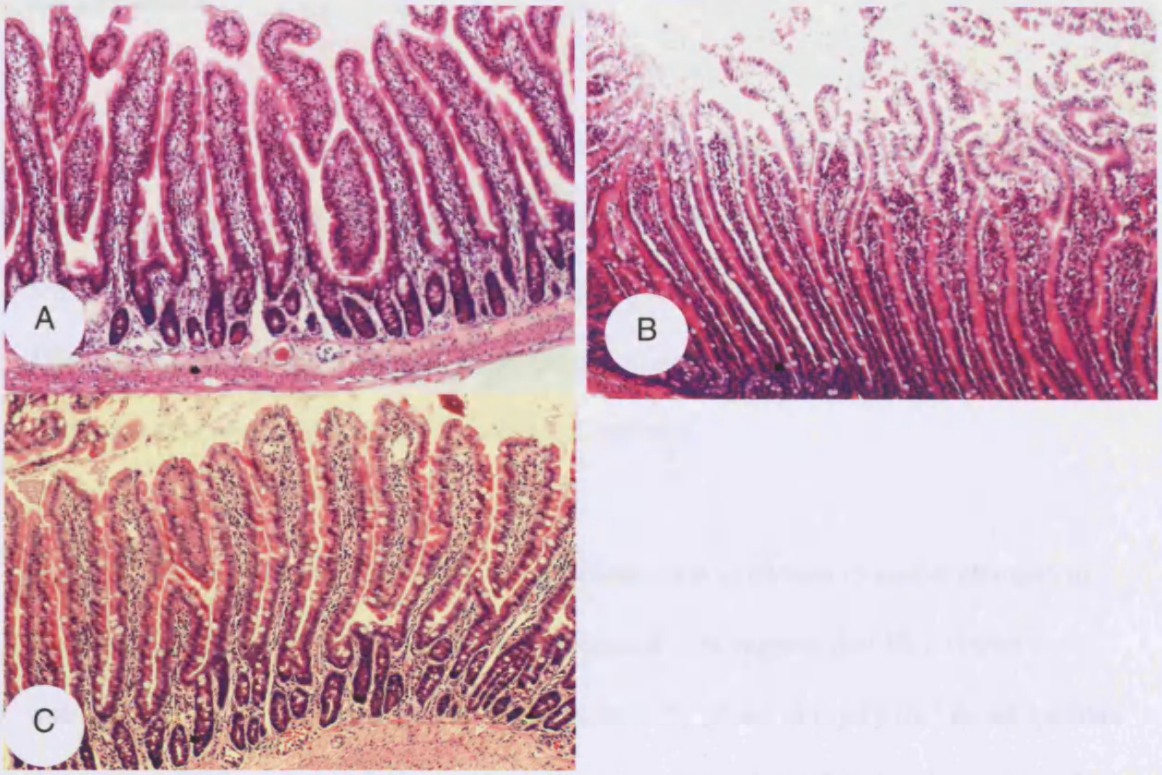


Figure 3.6.8 Representative photomicrographs of the ileum (A) in sham operated animals showing well preserved villi , (B) subjected to 30 min of ischaemia and 2 hr reperfusion (IR) showing disruption of villous pattern and (C) subjected to IPC + IR- depicting well preserved villi.

(H&E, original magnification x 100).

3.5 Discussion

The present study has clearly demonstrated that IPC improves the intestinal microvascular perfusion and oxygenation, while ameliorating IR injury. Serum LDH, which was used as a marker for intestinal injury (Sola *et al.*, 2001;Thompson *et al.*, 1990), reflected intestinal damage in IR group, whereas IPC decreased these levels. These results correlated with the histological analysis.

A period of 2 hrs of reperfusion following ischaemia was chosen to assess changes in the early phase of reperfusion injury. Experimental data suggest that IR induced intestinal injury occurs in a biphasic manner; an early phase of injury that develops over the course of 2 hrs of reperfusion and a later progressive phase that develops at 6 to 24 hrs after reperfusion (Slavikova *et al.*, 1998). Preconditioning times of 10 min ischaemia with 10 min reperfusion have been shown to protect against intestinal injury in experimental models of intestinal IR injury (Sileri *et al.*, 2004;Aksoyek *et al.*, 2002;Vlasov *et al.*, 2002).

LDF was used to measure the IMP. Following reperfusion, changes in IMP demonstrate that IR was characterised by no reflow phenomenon, which was abrogated by IPC. The increase of IMP within the initial 30 minutes of reperfusion with IPC suggests that the mechanism of preconditioning modulating flow in the microcirculation is an immediate phenomenon. Therefore, the effect of preconditioning is likely to involve modulation of immediate microcirculatory events at the level of capillaries and postcapillary venules, because these are the primary sites of IR-induced microcirculatory failure.

NIRS was used to measure the changes in intestinal tissue oxygenation at the level of capillaries. IR was associated with a decrease of HbO_2 and an increase of Hb, reflecting the dissociation of oxygen from haemoglobin, as oxygen is extracted by the intestinal tissue. These changes reflect reduced blood and oxygen supply to the tissue. The reduction in Cyt Ox reflects severe intracellular hypoxia and mitochondrial dysfunction. The failure of recovery of HbO_2 and a decline in Cyt Ox indicate persistent extracellular and intracellular hypoxia due to an inability to fulfil the oxygen demand with the reperfusion injury. In contrast, IPC was associated with an increase of both HbO_2 and Cyt Ox levels. This increase of Cyt Ox suggests that it is mitochondrial preservation that contributes to the mechanism of IPC.

Our results demonstrate that induction of ischaemia results in a steep rise of MABP. This may be mediated by a decrease in the baroreceptor input to the medullary vasomotor centre in response to reduced splanchnic perfusion (Hayward and Lefer, 1998). Reperfusion resulted in restoration of MABP to pre-ischaemic levels.

The portal venous flow did not show any sign of recovery despite reperfusion in the IR group. Turnage *et al.* demonstrated that the SMA blood flow does not return to normal on reperfusion but remains low in IR group (Turnage *et al.*, 1996). Thus reduced flow through the superior mesenteric vein would necessarily reduce portal venous flow. At the end of 2 hrs of reperfusion, IPC improved the portal venous flow significantly.

In summary, the data presented in this chapter showed that IR injury of the intestine results in profound derangement of intestinal microvascular perfusion, oxygenation and portal venous flow. IPC reversed these effects and attenuated the IR injury of the intestine.

Chapter 4 Protective effects of ischaemic preconditioning on the intestinal mucosal microcirculation and lungs following ischaemia reperfusion of the intestine

4.1. Introduction

In the previous chapter, we have shown that ischaemic preconditioning (IPC) improves the intestinal microvascular perfusion and tissue oxygenation. In this chapter, we investigate the effects of IPC on the red blood cell dynamics and leukocyte-endothelial interactions using intravital microscopy and examine the role of haem oxygenase. We also examine the effects of IR injury of the intestine and IPC on the lung histology. Intestinal tissue injury after a period of ischaemia is preferentially localised to the mucosal villi, with the underlying submucosal layers being virtually unharmed (Kong *et al.*, 1998). Microcirculatory failure showing impairment of capillary perfusion, activation and adhesion of leukocytes and eventual disruption of the mucosal barrier are the crucial hallmarks of IR injury in the mucosal villi (Kaminski and Proctor, 1989).

4.2. Materials and Methods

Animals were anaesthetised and operated upon as described previously in chapter 2.1.1.

4.3.1 Operative Procedures

Animals were anesthetized and operated upon Animals were anaesthetised and operated upon as described previously in chapter 2.1.1.

Laparotomy was carried out through a midline incision. The superior mesenteric artery (SMA) was identified and occluded with a non-traumatic vessel clamp to induce ischaemia. Reperfusion started when the clamp was released. In all animals, the

mucosal surface was exposed in a segment of exteriorized ileum by making a 30 mm incision along the anti-mesenteric border using an electric microcautery.

Following the period of reperfusion, there was macroscopic evidence of patchy necrosis along the length of the small bowel. Microscopically, the areas of necroses were associated with total stasis of blood flow. This patchy response is consistent with previous studies (Kalia *et al.*, 2002;Kong *et al.*, 1998). Because of this patchy response, the anti-mesenteric incision for the exposure of the mucosa was made in a relatively viable segment of the ileum which was associated with area of reflow in order to study the effects of IR injury and IPC. The animal's abdomen was covered with a plastic wrap (Saran wrap®, Dow Chemical, Michigan, USA) to prevent fluid evaporation. At the end of the experiment the animals were killed by exsanguination.

4.3.2 Experimental Protocol

Rats were randomly allocated to 3 study groups (n=12/group).

Group 1. Sham laparotomy, the SMA was identified with no vascular occlusion.

Group 2. IR, the SMA was occluded for 30 min, followed by a 2 hour period of reperfusion.

Group 3. IPC+IR, the SMA was occluded for 10 min and released for 10 min. This was followed by IR (as in Group 2).

In each group RBC dynamics were studied on 6 animals and the other 6 animals had evaluation of the interactions of leukocyte to the endothelium.

4.3.3 *In vivo* Fluorescence Microscopy

The animals were placed on the stage of a Nikon custom built microscope (Nikon, Japan) with an integrated heating system where the temperature was maintained at 37°C. The whole set up was placed on a pneumatic vibration isolation workstation (Newport, U.S.A) to minimise vibration. The small bowel was placed on to a specially designed plastic stage and a cover slip was then sited on to the mucosa. The microscope was illuminated with a 100 watt mercury arc lamp for epi-illumination fluorescence light microscopy. The microscope was equipped with water immersion 10 x and 40 x lenses. Images were acquired continuously using a charge-coupled device camera (CCD, JVC, Japan), displayed on to a high resolution monitor (Iiyama, Japan).The images were digitised by a video-frame grabber and then passed through to the central processing unit of a computer image workstation where they were analysed (Lucia image analysis software).

Following 30 min of reperfusion in the IR and IPC groups, or at equivalent time point in the sham group, at random 6 animals received fluorescein isothiocyanate (FITC) (Sigma, UK) labelled RBC (0.5 mL) intravenously in order to assess microvascular blood flow in the villi. FITC labelled RBC was prepared according to a modification of the method described previously (Zimmerhackl *et al.*, 1983) (See Appendix 2). Microscopically, the labelled cells showed a normal biconcave shape similar to native red cells. With the aid of FITC-labelled RBC, the following parameters were measured (a) mucosal perfusion index (MPI) (b) mean capillary diameter (MCD) and (c) the RBC velocity.

To evaluate the leukocyte-endothelial interactions in the mucosal villi, rhodamine 6G (0.2mL of 0.01%; Sigma UK) was injected to another 6 animals as a vital dye that stains the leukocytes avidly and accumulates in the mitochondria (Dunn *et al.*, 2002).

The microscopy technique does not allow the entire mucosa to be kept under observation, hence two areas were pre-selected in each animal, at a distance from each other for detailed surveillance. Recordings were made from these areas every 15 min after the administration of the fluorochromes for a period of 2 hours.

4.3.4 Mucosal Perfusion Index (MPI)

Microhemodynamics in the mucosa of the small intestine was evaluated by scanning the mucosa with the 10 x lens for semi-quanitative assessment of homogeneity and distribution of mucosal perfusion. 15-20 villi were allocated to three grades of perfusion (absent, irregular or good perfusion) according to the method described previously(Heuser *et al.*, 2000). A villus was considered to be 'poorly perfused' when at least 3-4 capillaries were not perfused over more than 20 seconds. Only villi without any perfusion were assigned to the 'absent' group.

The MPI was calculated by the formula:

$$MPI = (Ng + 0.5\ Np) / Nt$$

where Ng represents the number of well perfused villi, Np the number of poorly perfused villi and Nt the total number of villi analyzed. This index equals 1 if all the villi are well perfused and 0 if no villus is perfused at all (Fig 4.1).

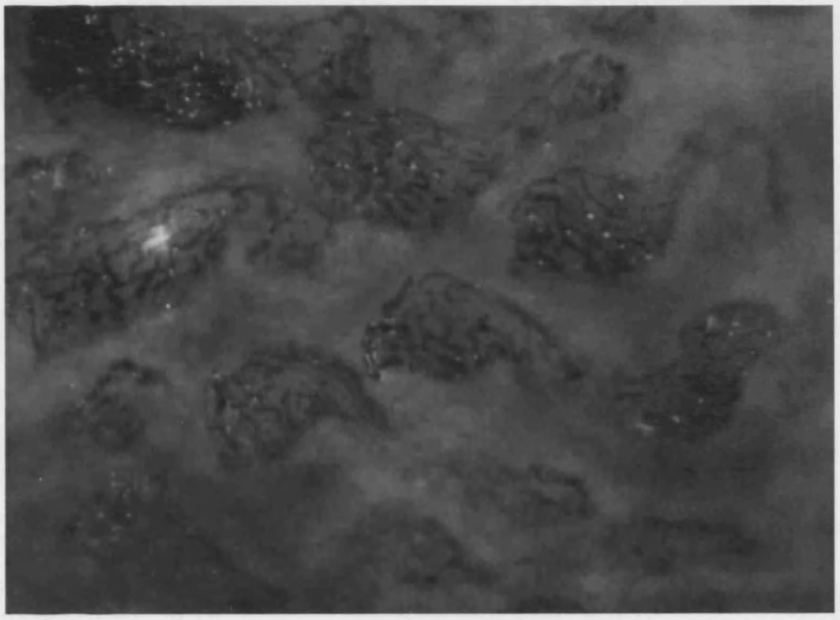


Figure 4.1. Villi stained with FITC labelled red cells (10x magnification).

4.3.5 Mean Capillary Diameter (MCD)

The 40x lens was utilised to assess the MCD. Only villi showing perfusion were chosen for the assessment of this parameter.15 capillaries randomized across the capillary network were chosen and the mean was then determined by offline analysis by Lucia image grabber.

4.3.6 Capillary Red blood cell velocity

RBC velocity was measured by the utilisation of the 40 x lens. 5 capillaries per villus were chosen and the velocity was determined by offline frame-to-frame analysis (Fig 4.2)

4.3.7 Quantification of Leukocyte Adhesion

The number of leukocytes adherent to the endothelium of up to four separate villi in two separate areas was determined by online analysis. A leukocyte was considered adherent if it do not move or detach from the endothelial lining within an observation period of up to 30 seconds. Data are expressed as the number of adherent leukocytes per length of villus at each time point. Leukocytes adherent within the blood vessel were often difficult to distinguish from those that had migrated to the interstitial space, but were still close proximity to the capillaries. Hence, both the groups of stationary leukocytes were regarded as adherent (Kalia *et al.*, 2002).

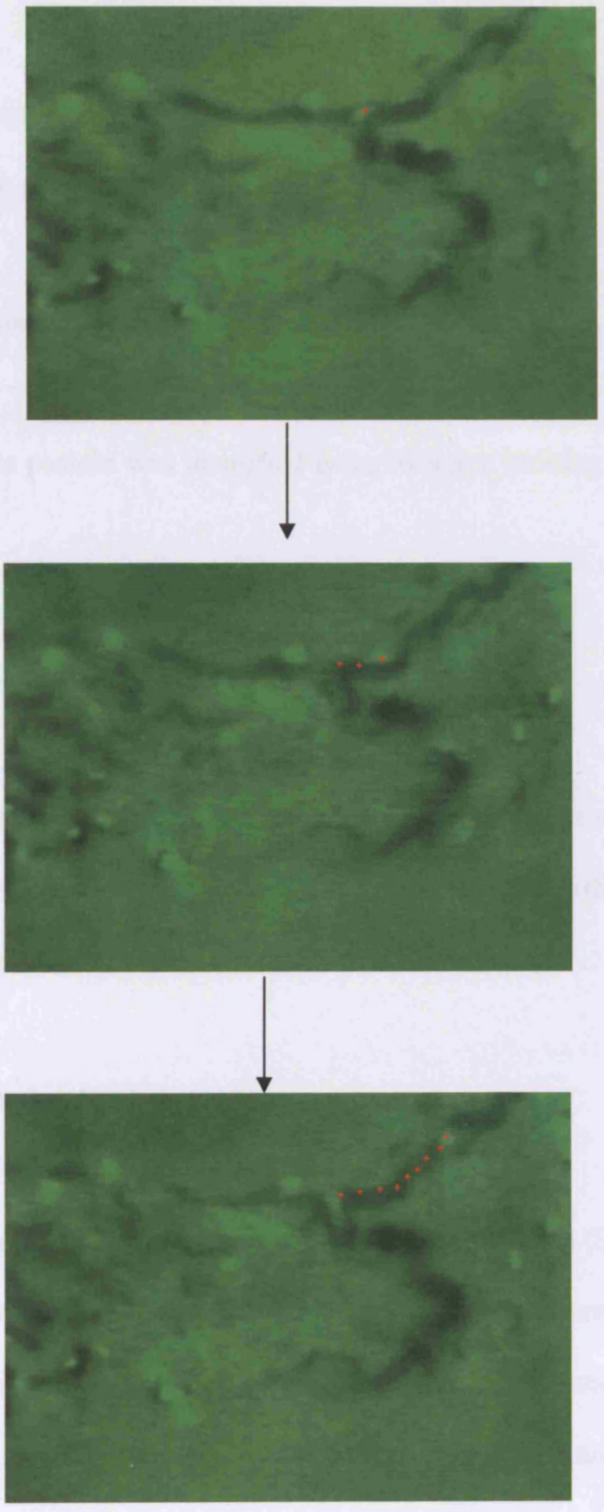


Figure 4.2.9 Frame by Frame analysis of measuring red blood cell velocity followed staining with red blood cells labelled with FITC.

4.3.8 Haem Oxygenase Activity Assay

HO activity in ileal microsomal fractions was measured using a spectrophotometric assay of bilirubin production as described previously in Chapter 2.6.1.

4.3.9 Western blot analysis

Haem Oxygenase protein was identified using Western blotting as described previously in chapter 2.6.2

4.3.10 Histological Investigation

At the end of the reperfusion period, samples of the lungs were removed, fixed in 10 per cent neutral buffered formalin and embedded in paraffin; 4μm thick sections were cut using a microtome and mounted on slides for H&E staining.

4.3.11 Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated, where unpaired Student's *t*-test was used for statistical analysis between groups. $P < 0.050$ was considered statistically significant.

4.4 Results

4.4.1 Mucosal Perfusion Index (MPI)

A homogenous microvascular perfusion with almost all villi being well perfused throughout the experiment was observed in sham operated animals. The mean MPI at the end of 2 hr reperfusion was 0.70 ± 0.04 (Fig 4.3).

In IR injury of the intestine, the mucosal perfusion was significantly decreased with many villi showing complete stasis. The mean MPI in the IR group at the end of 2 hr reperfusion was 0.40 ± 0.03 ($p<0.05$ vs sham). In the IPC group, there was a significant increase in the mucosal perfusion compared to IR. The MPI in IPC at the end of 2 hr reperfusion was 0.66 ± 0.04 ($p<0.05$ vs IR ; $p>0.05$ vs sham).There was complete absence of area of non-perfused villi (stasis).

4.4.3 Mean Capillary Diameter (MCD)

Fig 4.4 shows the trend in MCD during reperfusion in all the three groups. In sham operated animals the MCD at 15 min of virtual reperfusion point was 6.55 ± 0.80μm and was 6.80 ± 0.73μm at the end of the equivalent 2 hr reperfusion period. IR injury increased the MCD by 33% compared to sham reaching a value of 9.07 ± 0.56μm at 15 min of reperfusion. With reperfusion in IR group, MCD increased to 10.16 ± 0.12 μm at the end of the 2 hr reperfusion period. This increase of the capillary diameter in IR group from the start to the end of reperfusion coincided with the development of microvascular stasis. In the IPC group the mean capillary diameter at the end of 15 min of reperfusion was 6.94 ± 0.16 μm. However this was not significant compared to both sham and IR groups at this time point ($p>0.5$). The increase of capillary diameter in IR group on comparison to the IPC group reached statistical significance at 75 min of

reperfusion ($p<0.05$ IR vs. IPC). At the end of the 2 hr reperfusion period, the mean capillary diameter in the IPC group was 8.12 ± 0.34 μm ($p<0.05$ vs. IR).

4.4.4 Capillary Red Blood Cell Velocity

Fig 4.5 shows the changes in capillary RBC velocity in all the three experimental groups. Within the sham operated group the RBC velocity did not differ significantly during the experiment. In contrast, in the IR group RBC velocity decreased significantly compared with sham (0.25 ± 0.02 mm/s vs.0.60 ± 0.07 mm/s, $p<0.001$) at 15 min post reperfusion. IPC increased the RBC velocity to 0.67 ± 0.07 mm/s during the first15 min of reperfusion and reached a final value of 0.74 ± 0.2 mm/s at the end of the 2 hr reperfusion ($p<0.001$ vs. IR).

4.4.5 Leukocyte Adherence

In sham operated animals only occasional leukocytes were adherent within the endothelial lining of the mucosal villi throughout the experimental period (Fig.4.6). IR injury of the intestine induced a rapid, sustained and a significant increase in the leukocyte adhesion in the endothelium ($p<0.01$ versus sham; Fig. 4.7). It was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. IPC led to a significant decrease in the adhesion of the leukocytes to the endothelium ($p<0.01$ versus IR).

4..4.6 Haem Oxygenase analysis

Fig 4.8 depicts the mean ileal HO activity at the end of 2 hr of reperfusion in all the three experimental groups. The mean HO activity in the sham group was 409.66 ± 62.95 pico moles bilirubin/ mg/protein/hr. IPC lead to a two-fold increase of HO activity (1651.66 ± 231.92) as compared to IR (842.33 ± 85.12) ($p<0.01$ IPC vs. IR).Fig 4.9 shows the expression of HO in all the three groups as shown by western blot analysis.

4.4.7 Histological analysis

IR was associated with alveolar damage and neutrophilic infiltration in the lungs, whereas IPC attenuated this effect (Fig 4.10)

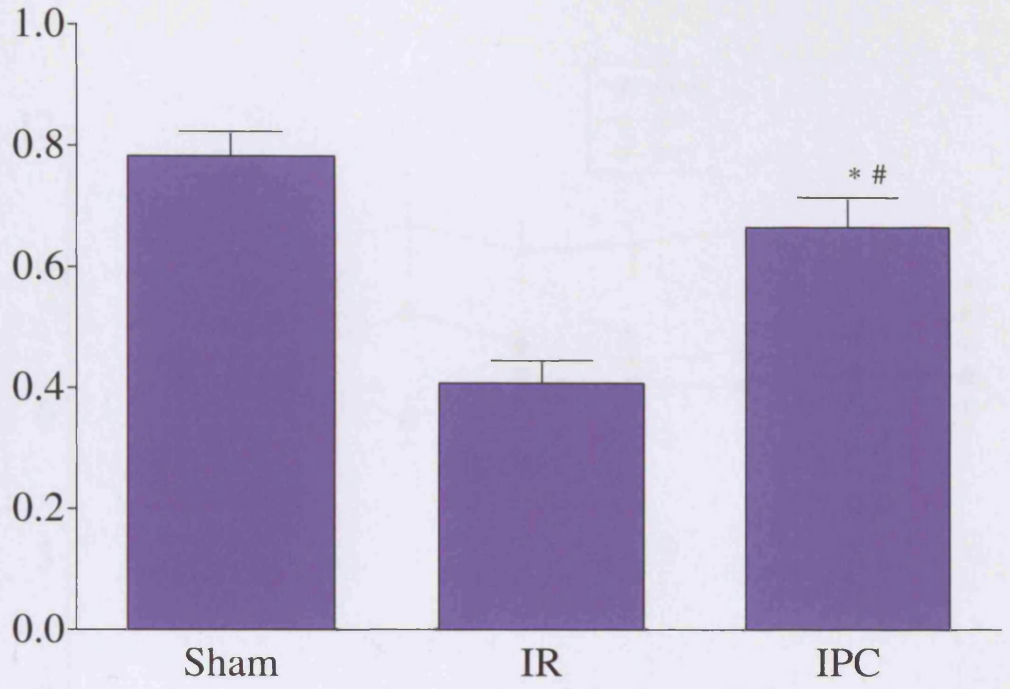


Figure 4.3. Homogeneity of perfusion in the mucosal villi expressed by mucosal perfusion index at the end of 2 hr reperfusion. For assessment of the perfusion pattern all villi were counted and divided into three groups according to quality of perfusion well, irregular and non-perfused.

Results are presented as mean ± SEM. (* $p<0.01$ vs IR; # $p>0.05$ vs sham).

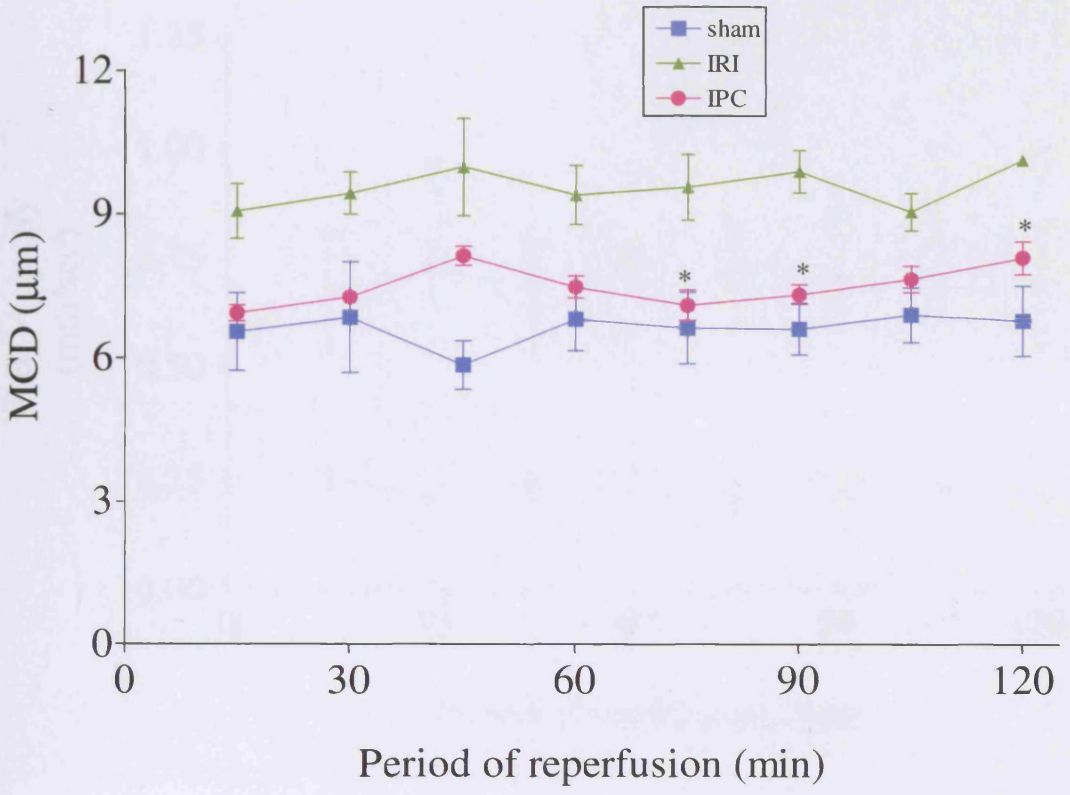


Figure 4.4. Mean Capillary diameter in all the three groups during the period of reperfusion. Results are presented as mean ± SEM of 6 animals from each group (*p<0.05 versus IR; **p<0.001 vs IR).

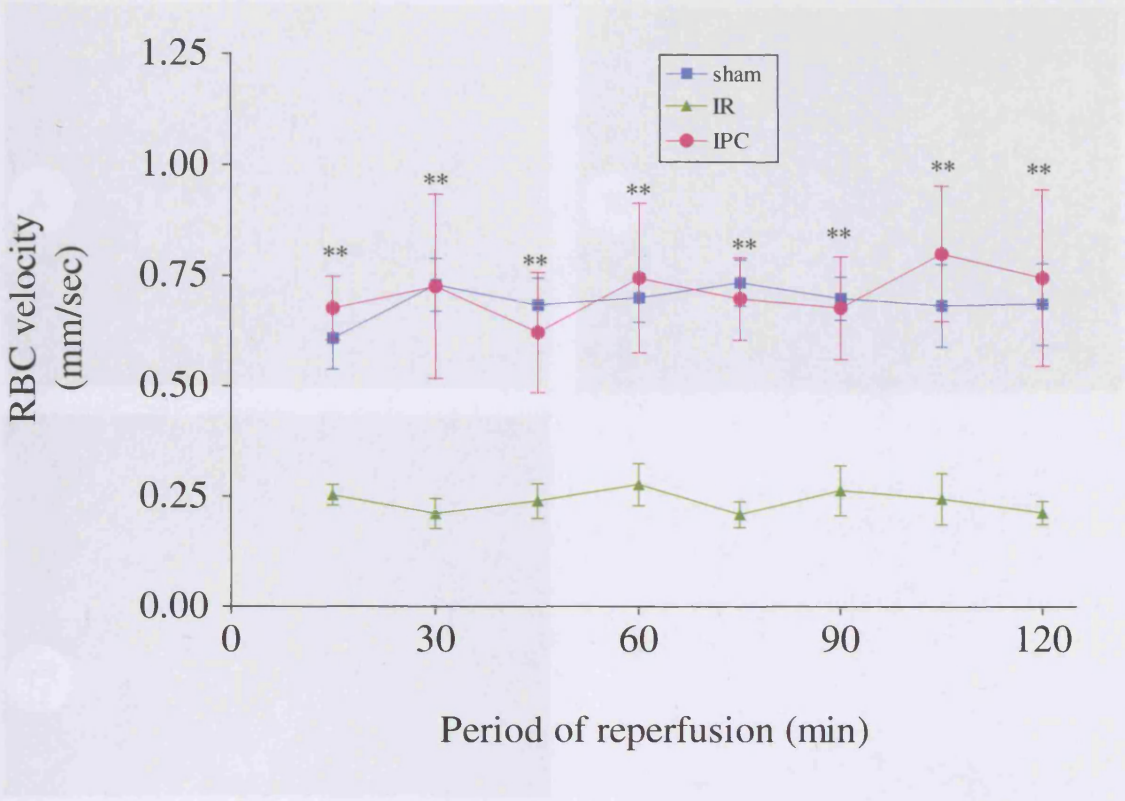


Figure 4.5.10. Red Blood cell velocity in all the three groups during the period of reperfusion. Results are presented as mean ± SEM of 6 animals from each group (*p<0.05 versus IR; **p<0.001 vs IR).

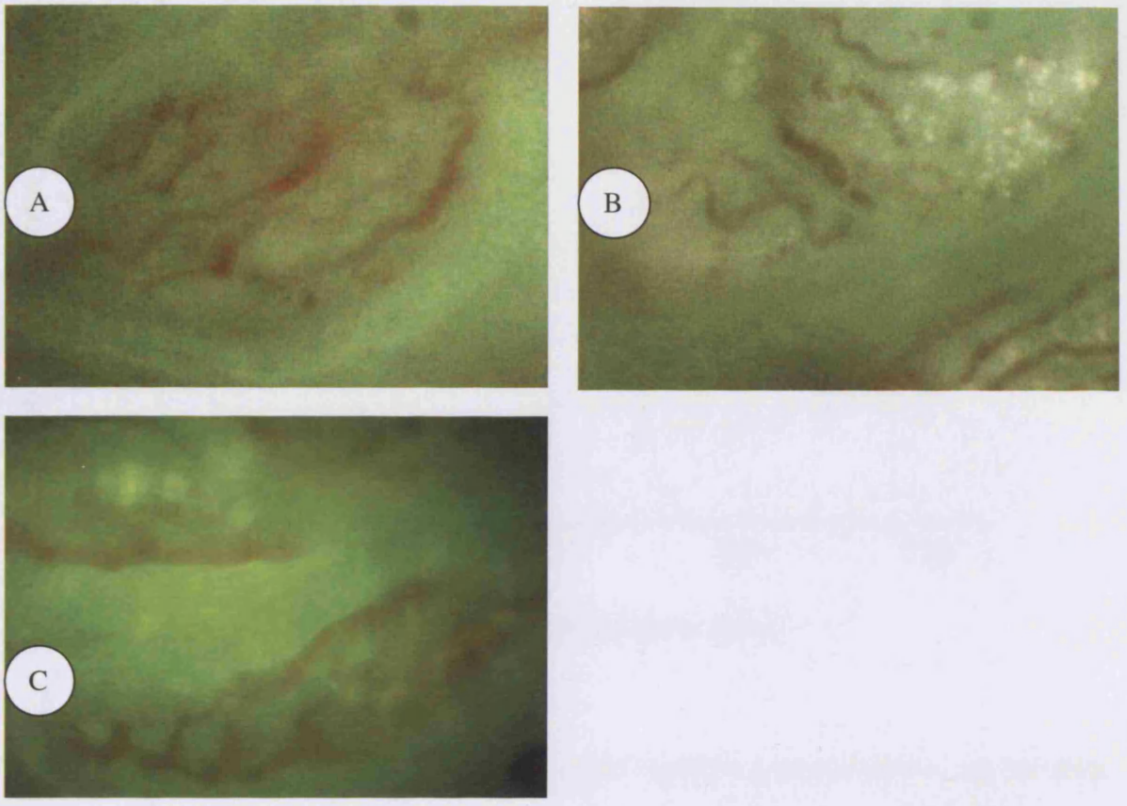


Figure 4.6. Leukocyte adherence in (A) sham, (B) IR injury and (C) IPC groups.

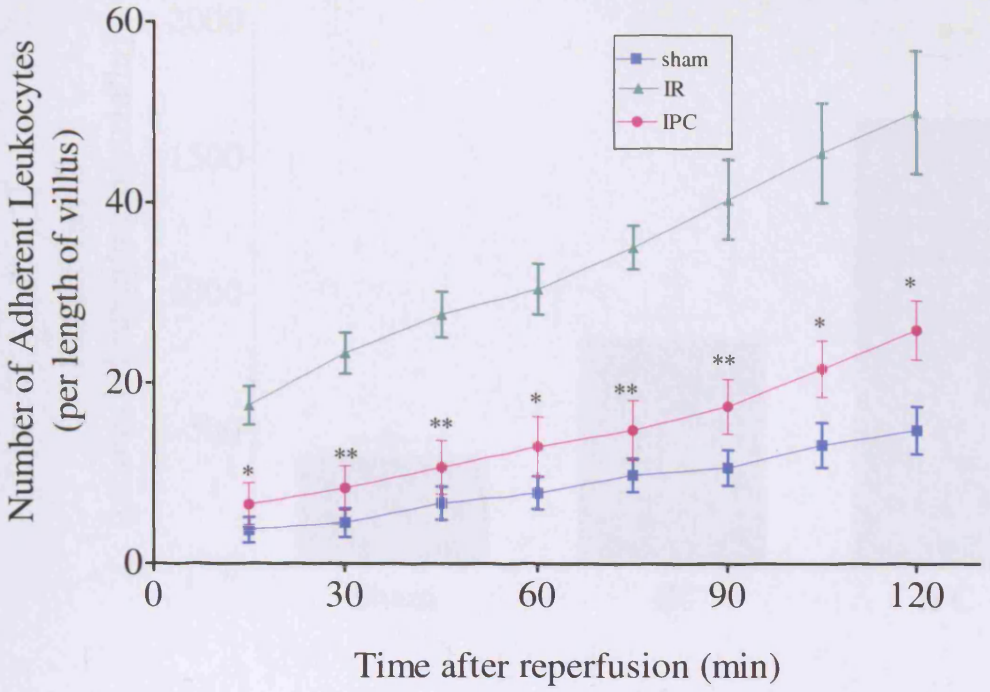


Figure 4.7. Number of adherent leukocytes in the capillary endothelium in all the three groups (cells/ mm^2). Results are presented as mean ± SEM of 6 animals from each group.(*$p<0.01$ versus IR ; **$p<0.001$ vs. IR)

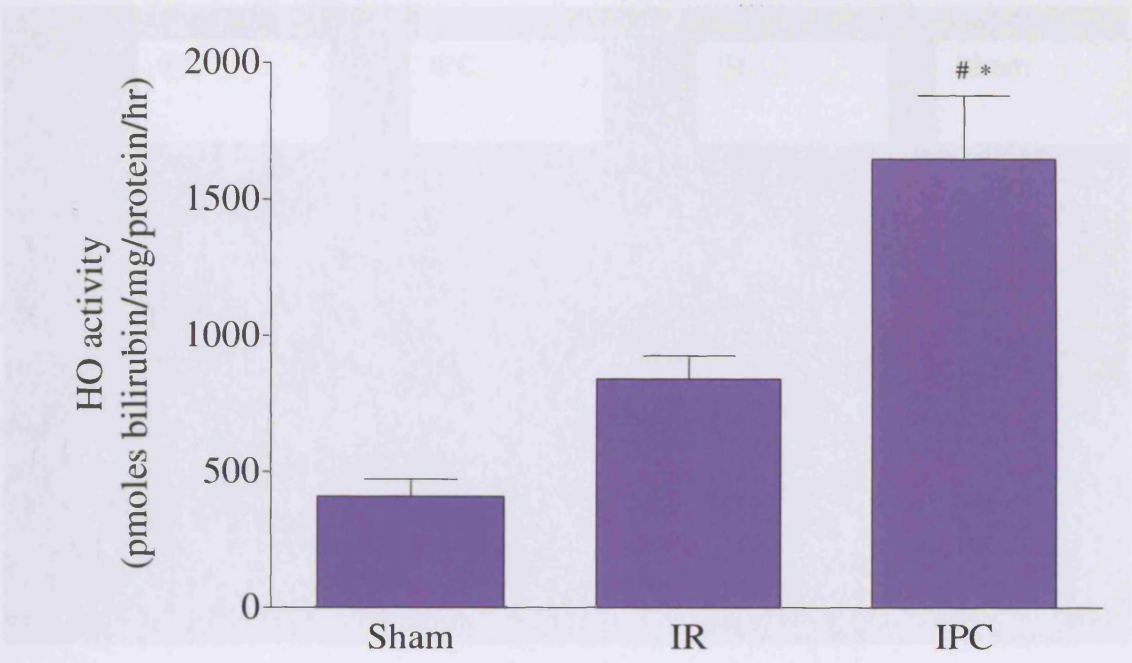


Figure 4.8. Ileal HO activity in all the three experimental groups at the end of 2 hr of reperfusion. (*p<0.01 IPC vs. IR; # p <0.01 sham vs. IR)

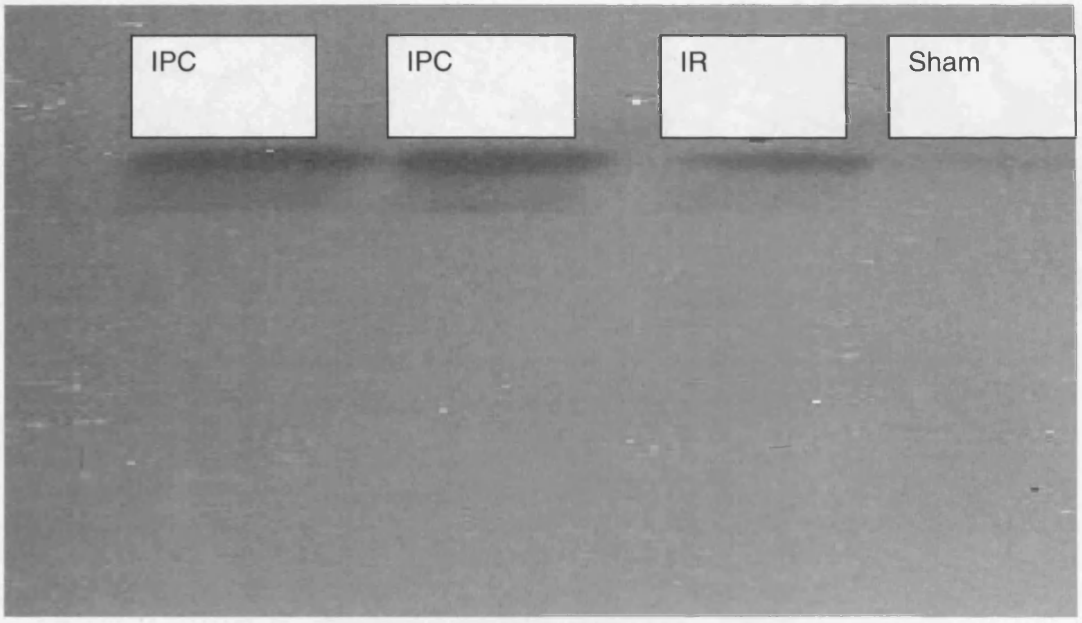


Figure 4.9. HO expression in all the three groups by western blot analysis.

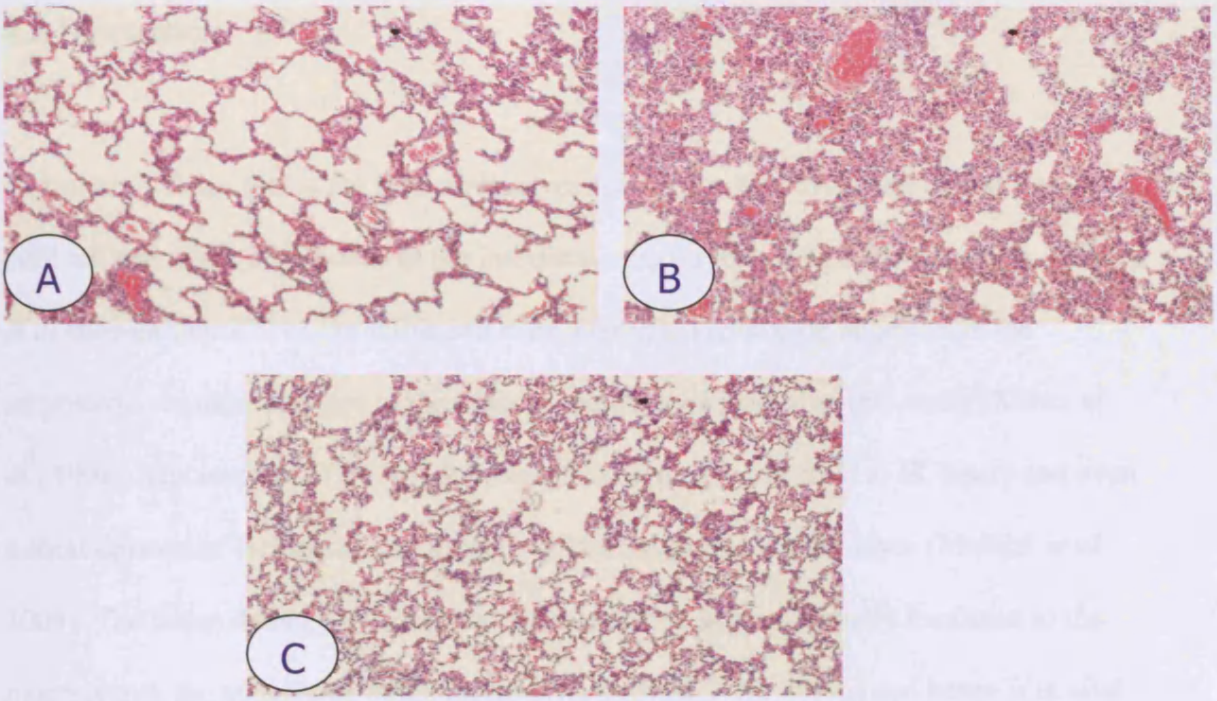


Figure 4.10.11. Representative photomicrographs of the lungs (A) in sham operated animals (B) subjected to 30 min of ischaemia and 2 hr reperfusion (IR) (C) subjected to IPC + IR. (H&E, original magnification x 100).

4.5 Discussion

To our knowledge this is the first study investigating the microvascular dynamics of both red and white blood cells in the intestinal mucosa following IPC. Although, Kubes *et al* showed that IPC of the feline intestine attenuated leukocyte adhesion in the mesenteric venules, changes in the mucosa were not addressed in this study (Kubes *et al.*, 1998). The mucosa of the small bowel is exceedingly sensitive to IR injury and even a short episode of ischaemia can induce substantial damage to the layer (Mallick *et al.*, 2004). The tissue damage in IR injury of the intestine is preferentially localized to the mucosa with the underlying submucosal layers virtually unaffected and hence it is vital to observe the changes in the mucosa. IR injury of the intestine was associated with decreased mucosal perfusion index, RBC velocity and increased leukocyte endothelial interactions in the mucosa. Our study has clearly demonstrated that IPC attenuates the microvascular disturbances by improving the mucosal perfusion index, RBC velocity and reduces the adhesion of leukocytes to the endothelium in the mucosal villi of small bowel mucosa following IR injury. IPC also preserved the mucosal villous architecture. These protective effects were clearly accompanied by a marked expression of HO.

The pathogenesis of cellular damage and microvascular damage following IR injury has yet to be fully elucidated. However, increased production of inflammatory mediators, decreased blood flow and vascular stasis are all implicated (Gonzalez *et al.*, 1994). Recent studies have shown that induction of HO helps to ameliorate tissue injury or inflammatory changes in a variety of experimental models (Attuwaybi *et al.*, 2004;Tamion *et al.*, 2002). However these studies do not address the role of HO in the intestinal microcirculation.

Capillary no-reflow and leukocyte endothelial interaction hallmark IR injury of the intestine(Kaminski and Proctor, 1989). Following IR injury, many cell types may be damaged by oxygen free radicals with RBC one of the most vulnerable (Chien, 1982). Impaired antioxidant defenses or increased production of oxygen free radicals may disturb the critical balance and result in oxidative damage to the RBC (Claster *et al.*, 1984). Injury to RBC is manifested both morphologically and physiologically (Chien, 1982). Since damaged RBC tend to increase their deformity and aggregate, the RBC velocity may decrease as demonstrated in this study. In addition, RBC lysis may occur, both within the capillary lumen and in the extracellular space, thereby releasing a source of free haem which may further exacerbate IR injury.

The results of the present study clearly demonstrate that IPC induced maintenance of adequate capillary perfusion *in vivo*, and indicate that the protective effect of the IPC phenomenon may be mediated by the action of HO, which catalyzes the conversion of haem to carbon monoxide (CO), biliverdin and free iron. CO and its action on capillary pericytes may play a major role in this protection. It is well known that capillaries are associated abluminally with pericytes which protrude primary cytoplasmic processes running along the axes of the capillaries (Hirschi and D'Amore, 1996). From these primary processes, lateral processes arise, which completely encircle the capillaries and form tight connections to the endothelial cells (Hirschi and D'Amore, 1996).These pericytes contain muscle cytoskeletal proteins, in particular α-smooth muscle actin which regulate microvascular blood flow (Hirschi and D'Amore, 1996). *In vivo* studies of liver tissue have shown that Ito cells, which are the sinusoid (liver capillary) associated pericytes, are primarily responsible for CO mediated blood flow regulation in sinusoids (Suematsu *et al.*, 1996).In addition, there is increasing evidence that CO inhibits the aggregation of platelets (Brune and Ullrich, 1987), which could result in

higher red blood cell velocity because of unhindered flow. Hence tissue viability increases because of improved nutritional supply and better elimination of toxic residues of oxidative stress. In a recent study Nakao et al demonstrated that inhalation of CO ameliorates IR injury in a model of bowel transplantation (Nakao *et al.*, 2003).

Biliverdin is subject to further degradation to bilirubin by the cytosolic enzyme biliverdin reductase (Maines, 1988). It acts as an antioxidant and is capable of scavenging oxygen free radicals that are thought to be primarily responsible for the tissue injury (Stocker *et al.*, 1987). Iron, the last product of haem breakdown, acts as an oxidant like other transition metals and catalyzes the formation of reactive hydroxyl radicals ($OH^{\bullet}$) by the Haber-Weiss reaction. Typically the $OH^{\bullet}$ causes biological damage by stimulating the free chain reaction known as lipid peroxidation, in which $OH^{\bullet}$ attacks the fatty acid side chains of the membrane phospholipids and causes organelle and cell disruption (Halliwell and Gutteridge, 1999). Therefore it seems to be of paramount importance to eliminate free iron from the tissue in order to maintain the cellular integrity after the stress event. To enable this process, an additional expression of ferritin, the iron-binding protein, is induced simultaneously by HO (Bauer and Bauer, 2002).

In IR injury it was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. Hayashi *et al.* demonstrated that overexpression of HO-1 in microvascular endothelial cells ameliorates oxidative injury and reduced the leukocyte endothelial interactions (Hayashi *et al.*, 1999).Hence, reduction of the leukocyte endothelial interaction by expression of HO-1 would reduce the plugging of the capillaries and thereby improve microvascular flow.

The profound effects on the lungs following IR injury of the intestine may be induced by the activation of neutrophils following translocation of bacterial endotoxins (Foulds *et al.*, 1997). It is possible that the increased mucosal permeability following ischaemia allows passive diffusion of endotoxin, which is a potent activator of neutrophils and could subsequently lead to remote multiple organ injury.

This study has shown that IR injury of the intestine induces rapid microcirculatory breakdown in addition to leukocyte adhesion in the mucosal layer with severe pathologic consequences. IPC of the intestine can maintain a functioning mucosal microcirculation and markedly attenuate the IR injury. IPC also attenuated the IR induced pulmonary injury.

Chapter 5 Pyrrolidine dithiocarbamate reduces ischaemia reperfusion injury of the small intestine

5.1. Introduction

In the previous chapter it was demonstrated that ischaemic preconditioning (IPC) induces the expression of haem oxygenase-1 and thereby alleviates ischaemia reperfusion (IR) injury. In this chapter we explore if pharmacological preconditioning (drugs that mimic IPC) can protect the small bowel from IR injury.

Therapeutic strategies aimed at ameliorating IR injury have focused both on preventing the effects of reactive oxygen species and on downregulating the signal transduction cascades related to the expression of proinflammatory genes. Pharmacologic preconditioning based on enhancing the production or activity of endogenous protective molecules has also been proposed as an alternative therapeutic intervention. Among such agents, pyrrolidine dithiocarbamate (PDTC) has a variety of biochemical activities, such as redox state alternation, chelation of heavy metals and enzyme inhibition (Tsuchihashi *et al.*, 2003). PDTC was initially regarded as a potent inhibitor of nuclear factor- kappa B (NF-κB) and used as an antioxidant compound to counteract the toxic effects of free radicals (Liu *et al.*, 1999). PDTC is one of the most effective inducers of haem oxygenase-1(HO-1), which also confers cytoprotection against oxidative stress (Hartsfield *et al.*, 1998).

To the best of our knowledge there are no previous studies exploring the effect of PDTC on IR injury of the intestine. Therefore, our study was designed to examine whether PDTC preconditioning induces HO expression in small intestine and reduces the inflammatory response during reperfusion by focussing on the intestinal microvascular perfusion and oxygenation.

5.3 Materials and Methods

5.3.1 Operative procedures

Animals were anaesthetised and prepared for laparotomy as described in previous chapter 2.1.1.

5.3.2 Experimental design

Rats were randomly allocated to 4 study groups (n=6/group).

Group 1. Sham laparotomy, the SMA was identified and passage of vicryl suture was performed, but without vascular occlusion.

Group 2. IR, the SMA was clamped for 30 min, followed by a 2 hour period of reperfusion.

Group 3. PDTC+IR. Animals received a single dose of 100 mg/kg of PDTC (Sigma Chemical Co, St Loius, Mo, USA) intramuscularly 30 min before IR.

Group 4. (ZnPP+ PDTC+ IR). ZnPP (Sigma Chemical Co, St Loius, Mo, USA) was dissolved in 0.2 mol/L sodium hydroxide and diluted in 0.9% sodium chloride. The dose of ZnPP was selected according to the experiment described by Tsuchihashi.(Tsuchihashi *et al.*, 2003) Animals received a single dose of 1.5mg/Kg of Zinc Protoporphyrin (HO inhibitor) given subcutaneously 30 min before PDTC administration.

5.3.3 Measurement of intestinal microvascular perfusion

Intestinal microvascular perfusion was measured by a surface laser Doppler flowmeter (LDF) (DRT4, Moor Instruments Limited, Axminster, UK) as described in chapter 3.

5.3.4 Measurement of intestinal tissue oxygenation

Intestinal tissue oxygenation was measured using NIRS as described in chapter 3.

5.3.5 Measurement of portal venous blood flow

We studied the effect of IR injury and PDTC on the portal venous blood flow (PVF). It was monitored continuously as described previously in chapter 3.3.5.

5.3.6 Haem Oxygenase Assay

HO activity in ileal microsomal fractions was measured as described previously in chapter 2.6.1.

5.3.7 Biochemical Assays

Blood samples were taken at the end of the experiments from the carotid artery. They were centrifuged at 2000 g for 10 min at room temperature to sediment the erythrocytes. The serum was removed and analysed on a Hitachi 747 auto-analyzer (Hitachi Ltd, Tokyo, Japan) by using commercially available enzymatic kits (Boehringer Mannheim Ltd., East Sussex,UK) for lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

5.3.8 Histological Investigation

At the end of the experiment, tissue samples of ileum were obtained as described previously in chapter 2.7 Ileal injury was assessed by a scoring system devised by Chiu *et al* under light microscopy without knowledge of study groups(Chiu *et al.*, 1970).

5.3.9 Data collection and statistical analysis

Data from the pulse oximeter, pressure transducer and LDF were fed into a laptop computer and collected continuously at a sampling rate of 2 Hz. The data were calculated as 1-min averages at baseline and every 30 min till the end of the experiment. All values are expressed as mean ± standard error of the mean (SEM). ANOVA and Bonferroni adjustment for multiple comparisons were used unless otherwise stated, where unpaired Student's *t*-test was used for statistical analysis between groups. $P< 0.05$ was considered statistically significant.

5.4 Results

5.4.1 Intestinal microvascular perfusion

IMP did not alter significantly during the course of the experiment in the sham group ($p >0.05$).There were significant differences between IR and PDTC groups in IMP at 30 min of reperfusion (46.7 ±2.8% in IR vs. 67.3±6.2% in PDTC, $p <0.010$). The increase in IMP persisted till the end of the 2 hr reperfusion period (42.5±2.8 % in IR vs. 69.1 ± 4.5 % in PDTC, $p<0.010$). ZnPP decreased the IMP to 37.3 ± 2.9 % at the end of 2 hr of reperfusion ($p<0.010$ vs. PDTC) (*Fig 5.1*).

5.4.2 Intestinal tissue oxygenation

Intestinal tissue oxygenation did not alter significantly during the course of the experiment in the sham laparotomy group (*Fig 5.2*). In IR group, with ischaemia there

was a significant decrease in HbO_2 and Cyt Ox with concomitant increase in Hb. There was a significant decline in HbO_2 and Cyt Ox levels following reperfusion compared to both the baseline and the ischaemia value.

PDTC was associated with significant increased levels of HbO_2 and Cyt Ox ($p<0.001$ IPC vs. IR).The levels of HbO_2 increased significantly on reperfusion and this increase persisted throughout the reperfusion period. The levels of Hb on reperfusion showed an increase from ischaemic levels, however, it was statistically lower compared to the IR group. ZnPP was associated with decreased HbO2 and Cyt Ox at the end of the reperfusion period. ($p<0.001$ vs. PDTC).

5.4.3 Portal venous blood flow

PVF was reduced significantly during the period of ischaemia ($p<0.001$ vs. baseline). At the end of the 2 hr reperfusion period it was improved significantly in PDTC group (42±9% vs. 69± 8 %; IR vs. PDTC respectively, $p<0.01$). ZnPP decreased the PVF to 36 ± 12 % of the baseline at the end of 2hr reperfusion ($p<0.01$ vs. PDTC) (*Fig 5.3*)

5.4.4 HO activity assay

The mean HO activity in the sham group was 409.66 ± 62.95 pico moles bilirubin/ mg/protein/hr. PDTC lead to a two-fold increase of HO activity (2085.83 ± 158.65) as compared to IR (768.66 ± 103.82) ($p<0.001$ PDTC vs. IR). The mean HO activity in the ZnPP group was significantly reduced as compared with PDTC group (94.00 ± 18.41) ($p<0.001$ vs. PDTC). (*Fig 5.4*).

5.4.5 Biochemical analysis

LDH was significantly reduced (P< 0.001) with PDTC (576.3 ± 98.7) as compared to IR (1866.0 ± 267.5) (*Fig 5.5*). The LDH value in sham group at the end of the reperfusion was 335.23 ± 77.7.

5.4.6 Histological analysis

IR and ZnPP resulted in a loss of normal mucosal villous pattern with increased neutrophils whereas PDTC ameliorated this effect. The mean histological score in IR was 3.0 ± 0.2 and with PDTC 1.5 ± 0.2 (# p<0.001 PDTC vs. IR). The mean histological score with ZnPP administration was 3.1 ± 0.3 († p <0.001 PDTC vs. ZnPP) (*Fig 5.6*).

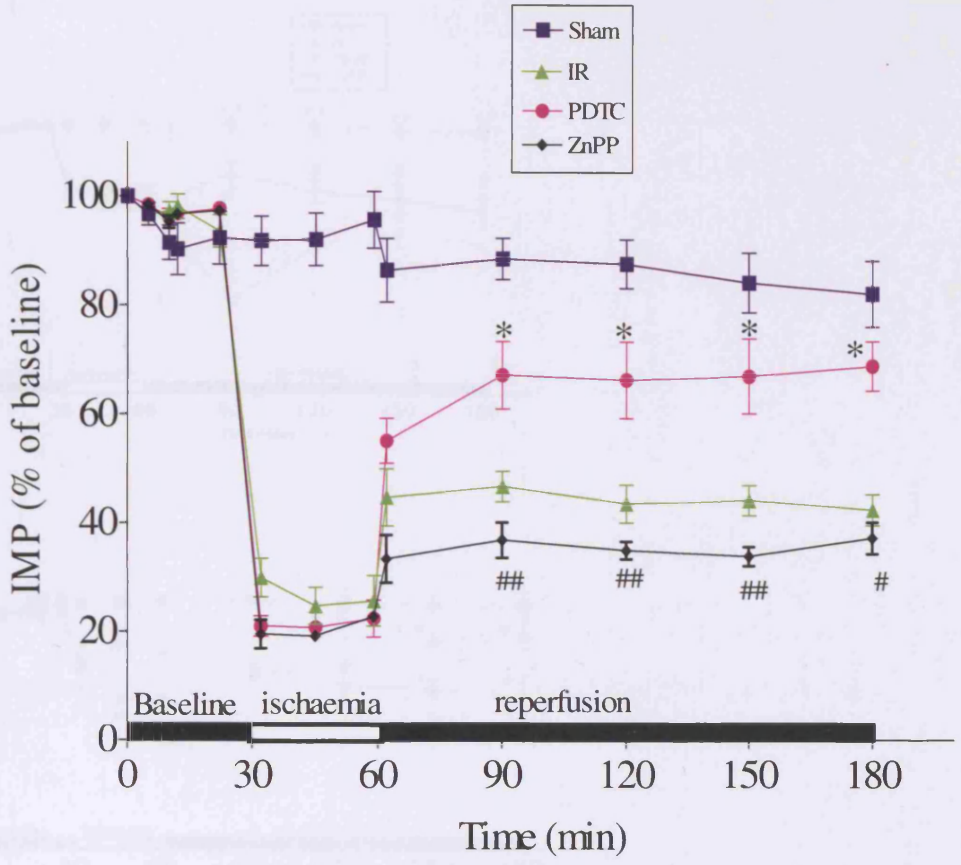


Figure 5.1. Intestinal microvascular perfusion (IMP) measured by LDF. For comparison, LDF readings were recorded as 100% at baseline. Values are mean ± SEM of 6 animals in each group.* $p<0.01$ PDTC vs. IR; ** $p<0.001$ PDTC vs. IR; # $p<0.01$ PDTC vs. ZnPP; ## $p<0.001$ PDTC vs. ZnPP)

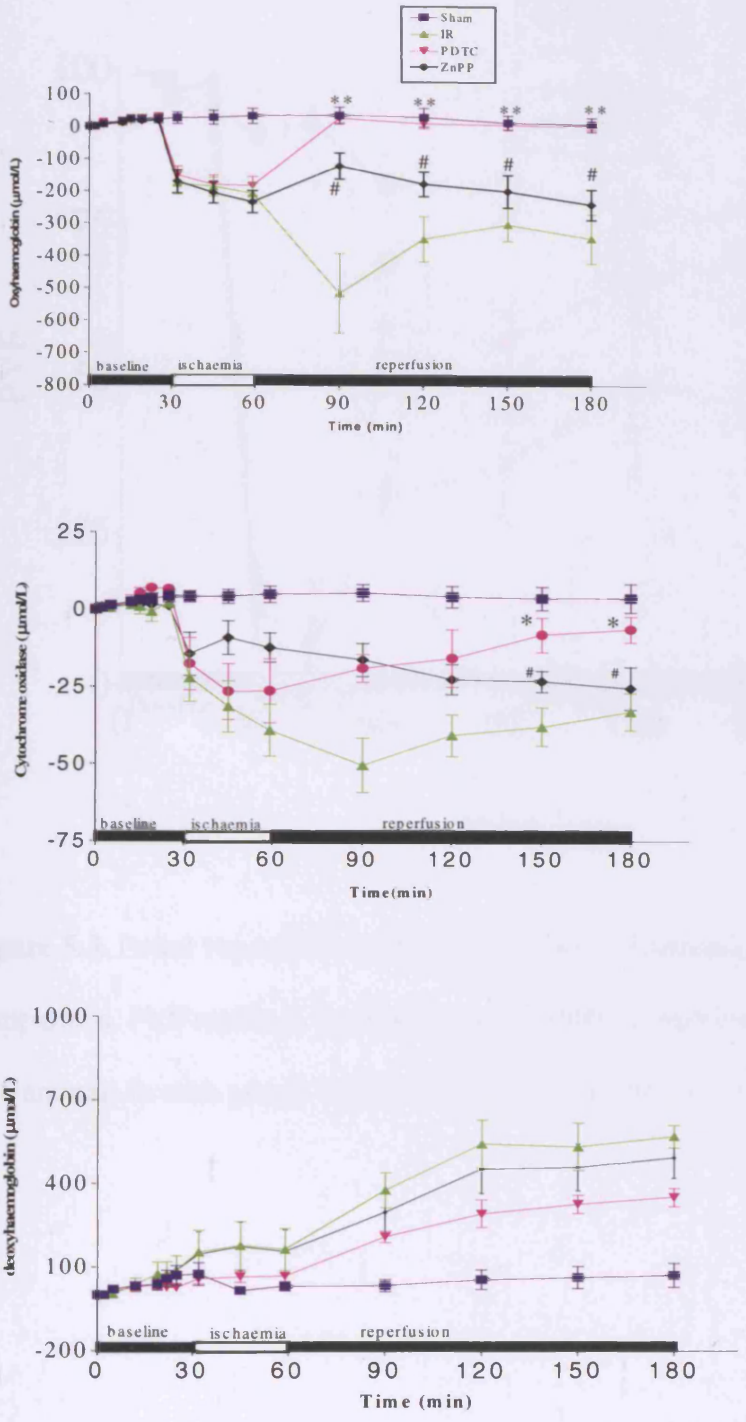


Figure 5.2. Intestinal tissue oxygenation as measured by NIRS. (* p<0.05 PDTC vs. IR; ** p<0.001 PDTC vs. IR; # p <0.05 PDTC vs. ZnPP; ## p <0.001 PDTC vs. ZnPP).

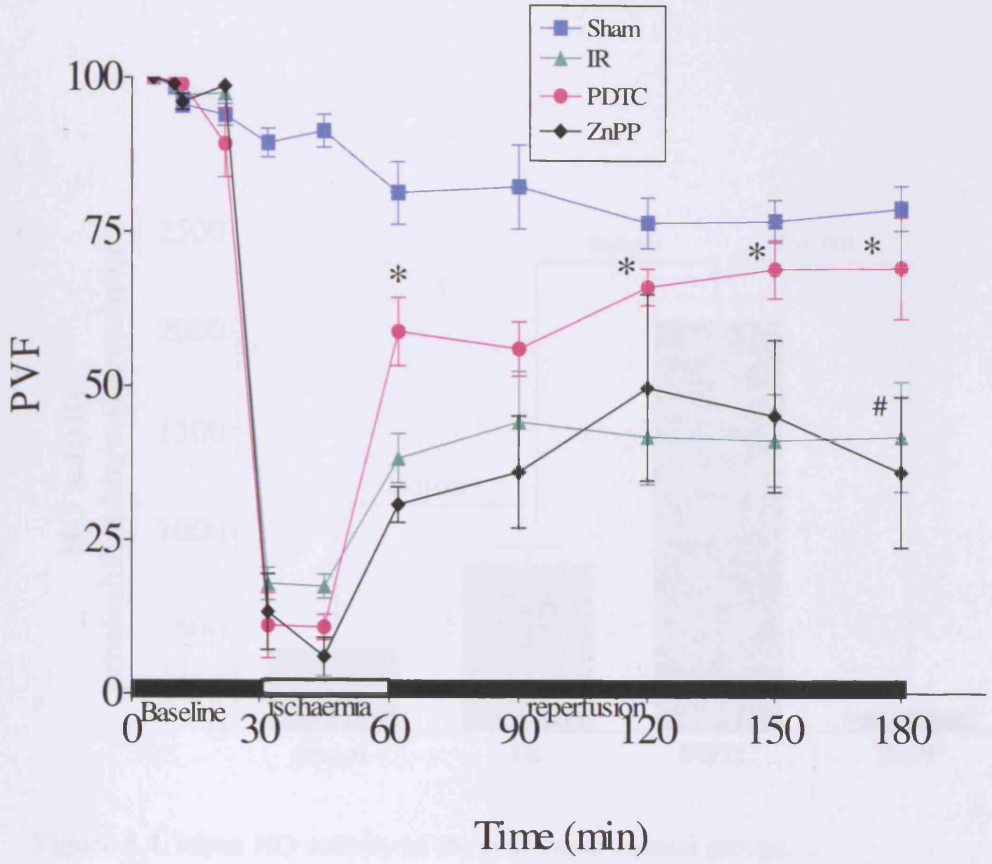


Figure 5.3. Portal venous blood flow measured by Transonic Doppler flow probe. For comparison, PVF readings were recorded as 100% at baseline. Values are mean ± SEM of 6 animals in each group. (*p<0.01 PDTC vs. IR; # p<0.01 PDTC vs. ZnPP)

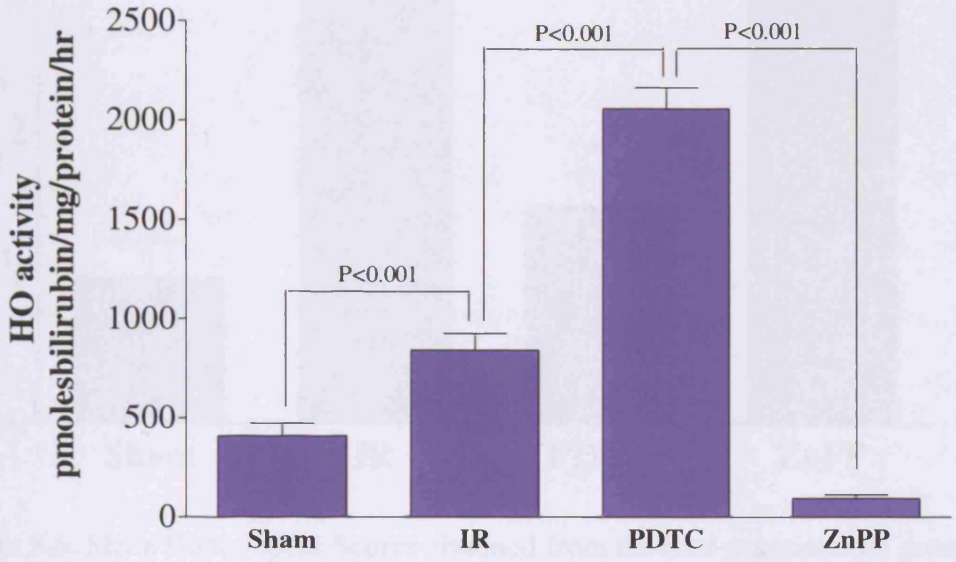


Figure 5.4. Mean HO activity in the four experimental groups.

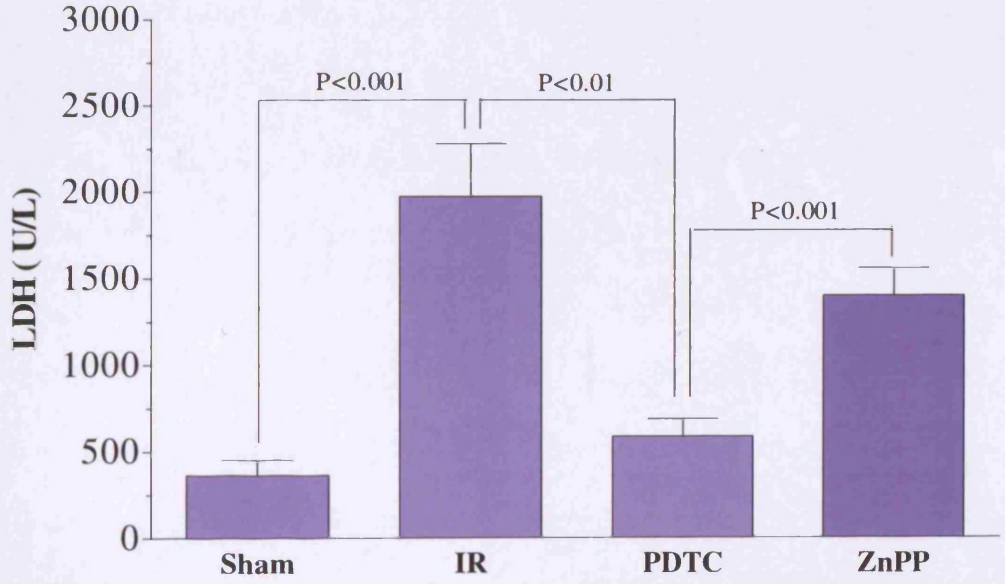


Figure 5.5. Serum LDH levels in the four experimental groups at the end of 2 hr period of reperfusion.

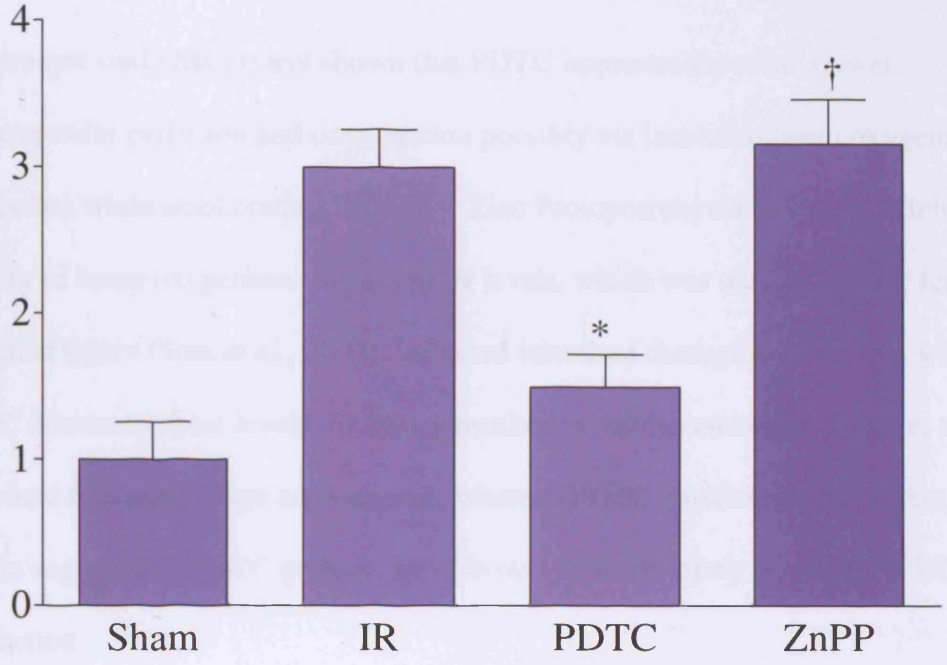


Figure 5.6. Mean Histological Scores obtained from the four experimental groups.

# p<0.001 PDTC vs. IR; † p<0.001 PDTC vs. ZnPP.

5.5 Discussion

The present study has clearly shown that PDTC improves the small bowel microvascular perfusion and oxygenation possibly via increased haem oxygenase expression while ameliorating IR injury. Zinc Protoporrphyrin was used to inhibit the activity of heme oxygenase. Serum LDH levels, which was used as marker for intestinal injury (Sola *et al.*, 2001), reflected intestinal damage in IR group, whereas PDTC decreased these levels. IR injury resulted in villous and crypt damage, which has attributed to haemorrhage and necrosis, whereas PDTC ameliorated this effect. These results suggest that PDTC protects small bowel from IR injury possibly via HO production.

One of the effective approaches for attenuating IR injury is to induce endogenous antioxidant genes such as HO-1. PDTC has been shown to be a potent inducer of HO-1 in many experimental models (Hartsfield *et al.*, 1998;Tsuchihashi *et al.*, 2003). Whereas increasing evidence suggests that HO-1 induction may mediate cellular protection against oxidant injury in both in *vitro* and in *vivo* models, the role of HO in the intestinal microcirculation is poorly understood. In this study the effects of PDTC against IR induced intestinal microcirculatory changes were studied by continuously measuring intestinal blood flow by using LDF, which has been used extensively to monitor intestinal blood flow in both animals and humans (Thollander *et al.*, 1997). In our study, the comparison of IMP between the groups at different time points shows that PDTC increased the IMP in the initial 30 minutes of reperfusion. This suggests that the mechanism of pharmacologic preconditioning by PDTC modulating flow in the microcirculation is an immediate phenomenon. Therefore, the effect of PDTC is likely to involve modulation of immediate microcirculatory events at the level of capillaries

and postcapillary venules, because these are the primary sites of microcirculatory failure induced by IR injury.

The results of the present study clearly demonstrate that PDTC induced maintenance of adequate microvascular perfusion and oxygenation *in vivo*, and indicate that this protective effect is mediated by the action of HO, which catalyzes the conversion of haem to CO, biliverdin and free iron. Clearly, the administration of ZnPP abolished the protective effect of PDTC.

The portal venous flow did not show any sign of recovery to baseline in spite of reperfusion in the IR group. Turnage *et al.* demonstrated that the superior mesenteric artery blood flow does not return to normal on reperfusion but remains low in IR group(Turnage *et al.*, 1996). Thus reduced flow through the superior mesenteric vein would necessarily reduce portal venous flow. At the end of 2 hr of reperfusion, PDTC improved the portal venous flow significantly.

In summary, the study in this chapter has demonstrated that intestinal IR injury induces rapid microcirculatory breakdown with tissue damage. Administration of PDTC maintains the intestinal microvascular blood flow and oxygenation, and markedly attenuates the IR injury. PDTC may be of particular value in preventing IR injury to the small intestine and might help to improve the results of small bowel transplantation. Intestinal IR injury is also an obligatory component of numerous surgical procedures. The results of the present study may therefore prove beneficial in many areas of clinical research. Additional studies are clearly warranted to evaluate the clinical efficacy of PDTC in the prevention of IR injury of the small intestine.

Chapter 6 Effects of Pyrrolidine dithiocarbamate on the mucosal villous microcirculation following ischaemia reperfusion injury of the intestine

6.1 Introduction

In the previous chapter we showed that pyrrolidine dithiocarbamate (PDTC) improves the intestinal microvascular perfusion and oxygenation and thereby attenuates small intestinal IR injury. In this chapter we investigate the effects of PDTC on the small bowel microcirculation by focussing on the RBC dynamics and leukocyte endothelial interactions. We also examined if PDTC pretreatment on pulmonary tissue injury after intestinal I/R had any effect.

6.2 Materials and Methods

Animal care and experimental protocols were performed as described in previous chapters.

6.2.1 Operative Procedures

Animals were anesthetized and subjected to laparotomy as described in previous chapters.

6.2.2 Experimental Protocol

Rats were randomly allocated to 4 study groups (n=12/group).

Group 1. Sham laparotomy, the SMA was identified with no vascular occlusion and animals received a single dose of 2mL/ kg of 0.9% normal saline subcutaneously.

Group 2. IR, the SMA was occluded for 30 min, followed by a 2 hour period of reperfusion.

Group 3. PDTC+IR. Animals received a single dose of 100 mg/kg of PDTC (Sigma Chemical Co, St Loius, Mo, USA) dissolved in 0.9% sodium chloride given

subcutaneously 30 min before IR. The time course and dose of PDTC used were based on previous studies by Liu *et al* (Liu *et al.*, 1999).

Group 4. (ZnPP+ PDTC+ IR). ZnPP (Sigma Chemical Co, St Loius, Mo, USA) was dissolved in 0.2 mol/L sodium hydroxide and diluted in 0.9% sodium chloride. The dose of ZnPP was selected according to the experiment described by Tsuchihashi (Tsuchihashi *et al.*, 2003). Animals received a single dose of 1.5mg/Kg of ZnPP subcutaneously 30 min before PDTC administration.

In each group RBC dynamics were studied on 6 animals and the other 6 animals had evaluation of the interactions of leukocyte to the endothelium.

6.2.3 *In vivo* Fluorescence Microscopy

The animals were placed on the stage of a Nikon custom built microscope and subjected to intra vital fluoroscopic examination as described in the previous chapters.

6.2.4 Mucosal Perfusion Index (MPI)

MPI was evaluated as described previously in chapter 4.3.4.

6.2.5 Capillary Red blood cell velocity

RBC velocity was measured as described previously in chapter 4.3.5.

6.2.6 Quantification of Leukocyte Adhesion

The number of leukocytes adherent to the endothelium of up to four separate villi in two separate areas was determined by online analysis as described previously in chapter 4.3.7.

6.2.6 Western blotting for HO-1

Ileal samples were also analyzed by the western immunoblotting technique as described previously.

6.2.7 Biochemical Assays

Blood samples were taken after two hours reperfusion from the carotid artery and analyzed as described previously in chapter 3.3.6.

6.2.8 Histological Investigation

At the end of the reperfusion period, samples of were removed, fixed in 10 per cent neutral buffered formalin and embedded in paraffin; 4μm thick sections were cut using a microtome and mounted on slides for haematoxylin and eosin staining.

6.2.9 Statistical Analysis

All data are expressed as mean ± standard error of mean (SEM) values. ANOVA and Bonferroni adjustment for multiple comparisons was used for statistical analysis. Unpaired Student's *t* test was used for comparison between two groups. $P < 0{\cdot}05$ was considered statistically significant.

6.3 Results

6.3.1 Systemic Haemodynamic Parameters

There were no significant changes in HR and SaO_2 throughout the experiment in all the four experimental groups ($p>0.1$). In sham group, the MABP did not change significantly throughout the experiment ($p >0.05$). Clamping of the SMA was associated with a significant increase in MABP. Reperfusion restored MABP to pre-ischemic values. However, the MABP did not change significantly during the reperfusion period in all the four groups (*Table 6.1*).

6.3.2 Mucosal Perfusion Index (MPI)

A homogenous microvascular perfusion with almost all villi being well perfused throughout the experiment was observed in sham operated animals. The mean MPI at the end of 2 hr reperfusion was 0.70 ± 0.04 (*Fig 6.1*).

In IR injury of the intestine, the mucosal perfusion was significantly decreased with many villi showing complete stasis. The mean MPI in the IR group at the end of 2 hr reperfusion was 0.40 ± 0.03 ($p<0.05$ vs. sham). In the PTDC group, there was a significant increase in the mucosal perfusion compared to IR. The MPI in PDTC at the end of 2 hr reperfusion was 0.68 ± 0.04 ($p<0.05$ vs. IR; $p >0.05$ vs. sham).There was complete absence of areas of non-perfused villi (stasis). ZnPP was associated with decreased mucosal perfusion index of 0.33 ± 0.04 ($p<0.05$ vs. PDTC).

6.3.3 Capillary Red Blood Cell Velocity

Within the sham operated group the RBC velocity did not differ significantly during the experiment. On the contrary, in the IR group RBC velocity decreased significantly compared with sham (0.25 ± 0.02 mm/s vs.0.60 ± 0.07 mm/s, $p<0.001$) at 15 min post reperfusion. PDTC increased the RBC velocity to 0.71 ± 0.10 mm/s during the first15 min of reperfusion and reached a final value of 0.80 ± 0.23 mm/s at the end of the 2 hr reperfusion ($p<0.05$ vs. IR). The administration of ZnPP was associated with decreased RBC velocity of 0.27 ± 0.04 mm/ s at the end of the 2 hr reperfusion ($p <0.05$ vs. PDTC).(*Fig 6.2*)

6.3.4 Leukocyte Adherence

IR injury of the intestine induced a rapid, sustained and a significant increase in the leukocyte adhesion in the endothelium ($p<0.01$ versus sham; *Fig.6.3*). It was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. PDTC led to a significant decrease in the adhesion of the leukocytes to the endothelium ($p<0.05$ vs. IR). ZnPP administration led to increase in the adhesion of leukocytes at the end of 2 hr reperfusion ($p<0.05$ vs. PDTC).

6.3.5 Western blotting for HO-1

The administration of PDTC was associated with increased expression of HO-1 whereas ZnPP abolished this effect. (Fig 6.4 A& B).

6.3.6 Lung Histology

The alveoli were collapsed in IR and ZnPP groups. The capillaries within the interstitium were often congested with red blood cells and a marked neutrophilic infiltrate was apparent in IR and ZnPP groups, indicating haemorrhage. PDTC prevented any injury to the pulmonary tissue and the lung histology was similar to that in sham, with numerous distended alveoli with thin flattened and delicate alveolar walls. The alveoli were well aerated and few neutrophils were observed within the interstitium (*Fig 6.5*).

	Baseline	30 mins of ischaemia	1 hr perfusion	2 hrs of Reperfusion
Heart rate				
Sham	250 ± 5	252 ± 3	240 ± 8	230 ± 10
IR	251 ± 2	252 ± 2	220 ± 4	227 ± 12
PDTC	252 ± 3	251 ± 3	240 ± 10	232 ± 7
ZnPP	245± 10	236 ± 5	242 ± 8	241± 5
SaO_2				
Sham	98 ± 2	95 ± 2	94 ± 3	95 ± 2
IR	98 ± 1	95 ± 2	97 ± 2	95 ± 3
PDTC	98 ± 1	96 ± 3	93 ± 1	96 ± 3
ZnPP	98 ± 2	97 ± 2	95 ± 2	96 ± 2
MABP				
Sham	83 ± 6	76 ± 5	74 ± 6	78 ± 8
IR	90 ± 3	108 ± 5*	81 ± 3	72 ± 5
PDTC	87 ± 5	107 ± 5*	79 ± 4	73 ±6
ZnPP	83 ± 3	104 ± 2*	82 ± 5	76 ± 3

Table 6.1. Systemic haemodynamic parameters in all the four groups. Key: HR- Heart Rate (in beats per minute); SaO_2- arterial oxygen saturation (in %); MABP- Mean arterial blood pressure (in mm Hg). * $p < 0.01$ vs. baseline.

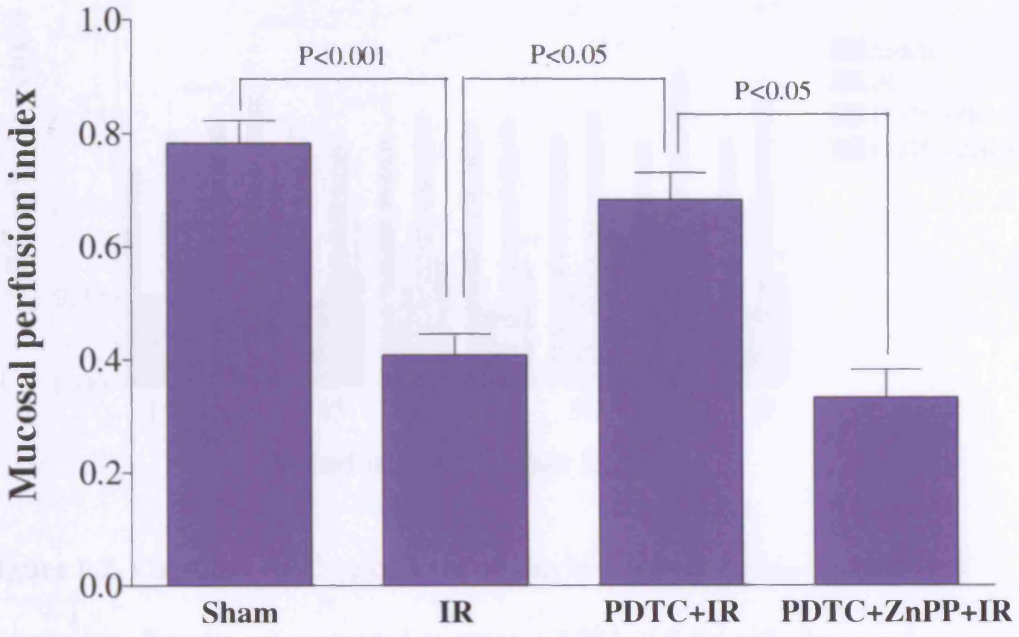


Figure 12.1 Mucosal perfusion index at the end of 2 hr reperfusion in all the four experimental groups. For assessment of the perfusion pattern all villi were counted and divided into three groups according to quality of perfusion well, irregular and non-perfused.

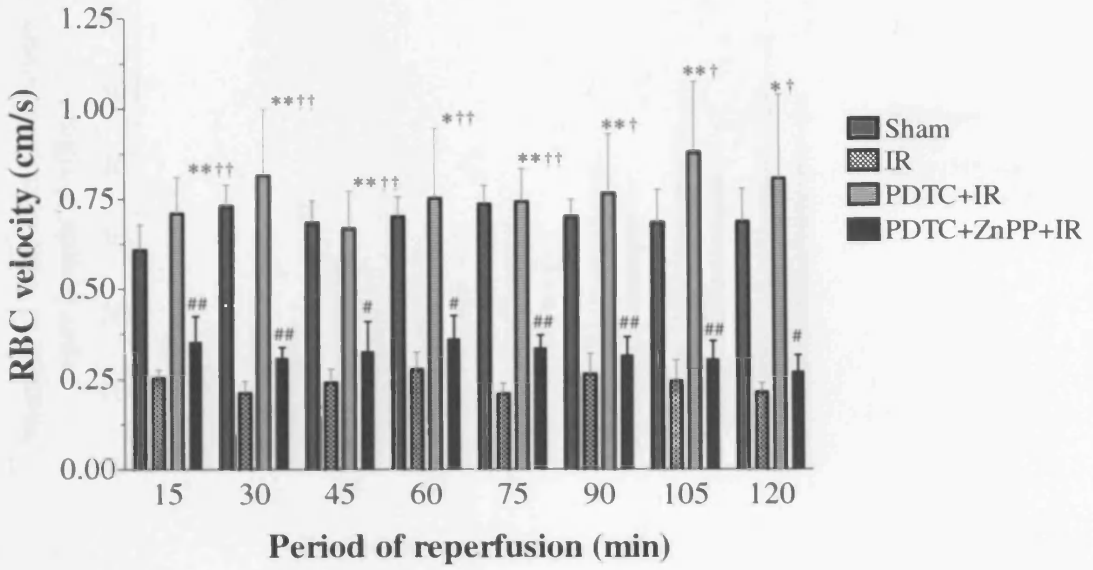


Figure 6.2. Capillary RBC velocity in all the four groups during the period of reperfusion. Results are presented as mean ± SEM of 6 animals from each group (*p<0.05 PDTC vs. IR; **p<0.01 PDTC vs. IR; # p<0.05 ZnPP vs. PDTC; ## p<0.01 ZnPP vs. PDTC; †p<0.05 IR vs. Sham; †† p<0.001 IR vs. Sham).

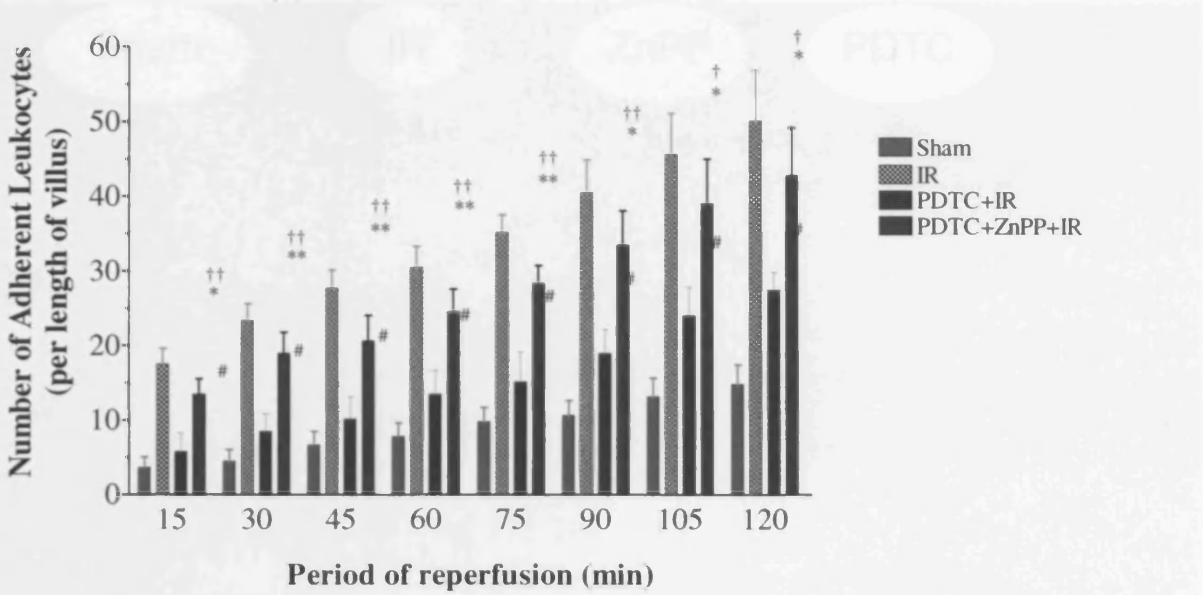
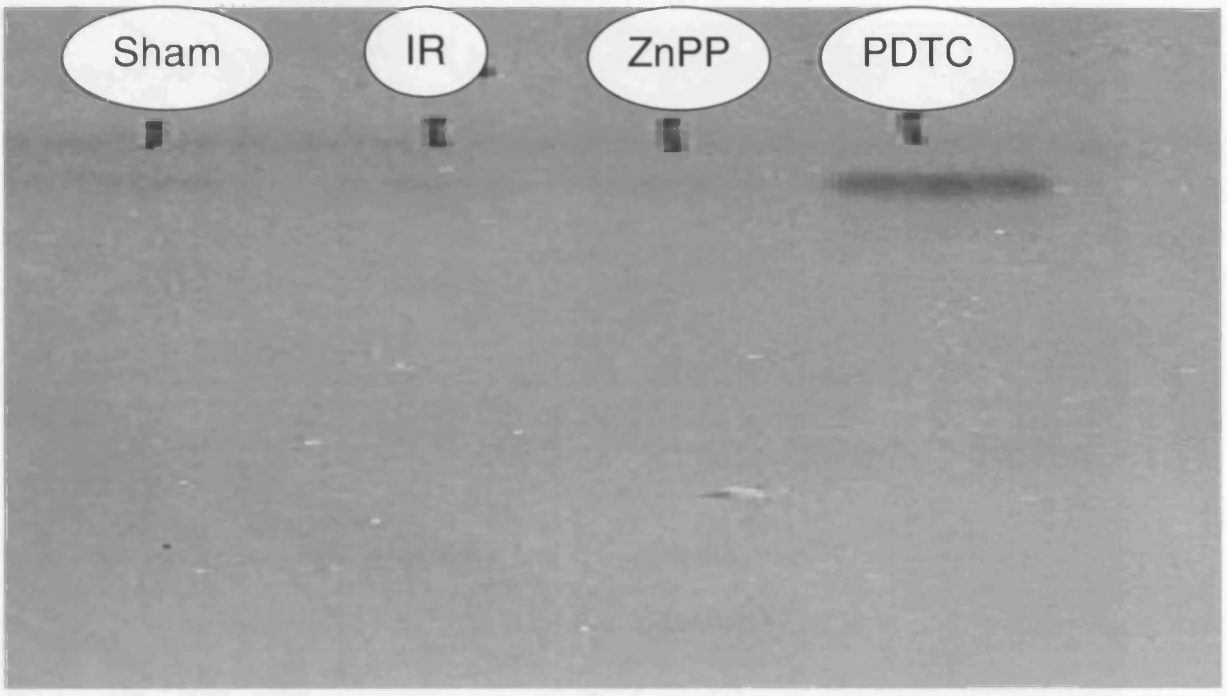


Figure 6.3. Number of adherent leukocytes in the capillary endothelium in all the four groups. Results are presented as mean ± SEM of 6 animals from each group.(*p<0.05 PDTC vs. IR ; **p<0.001 PDTC vs. IR; # p<0.05 ZnPP vs. PDTC; ## p<0.01 ZnPP vs. PDTC; † p<0.01 IR vs. Sham; †† p<0.001).



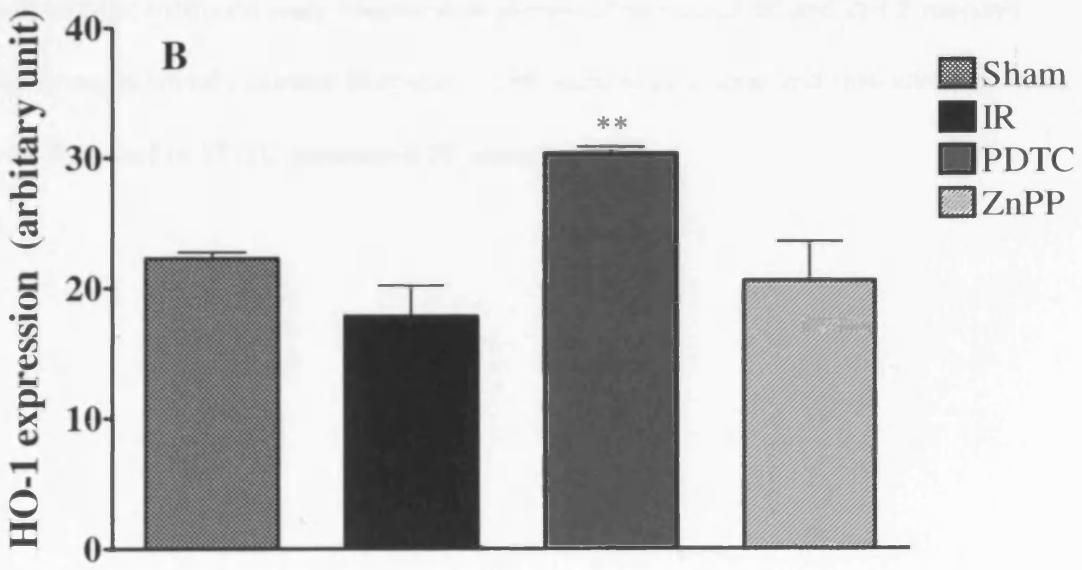


Figure 6.4 A & B. Western blotting for HO-1 in the four experimental groups. (** $p<0.001$ PDTC vs. IR & ZnPP).

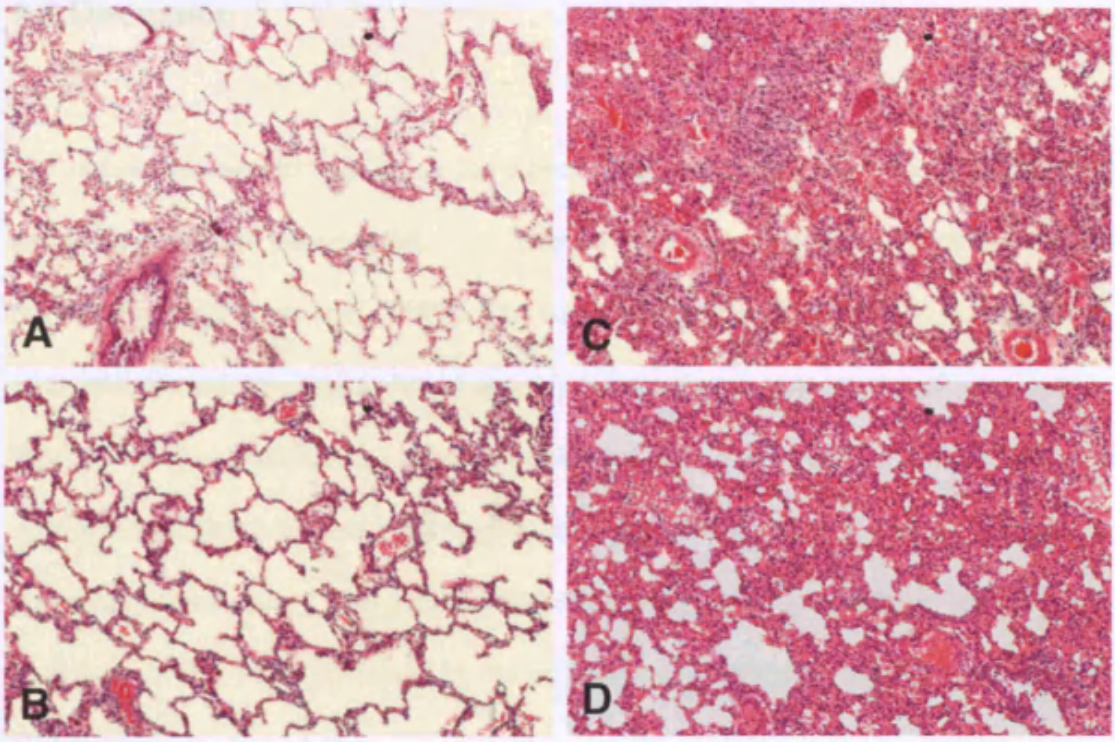


Figure 6.5. Lung histology of animals in (A) sham laparotomy, (B) IR ,(C) PDTC+IR and (D) ZnPP groups. Collapsed alveoli, thickened interstitial walls, and a dense neutrophilic infiltrate were observed in untreated intestinal IR and ZnPP animals. However, relatively normal histology, with numerous alveoli and thin alveolar walls, was observed in PDTC pretreated IR animals.

6.5 Discussion

Gut ischaemia and mucosal injury have been proposed to play a role in the development and maintenance of the systemic inflammatory response, a key factor causing multiple organ failure (Pastores *et al.*, 1996). Our study has clearly demonstrated that PDTC attenuates the microvascular disturbances by improving the mucosal perfusion index, RBC velocity and reduces the adhesion of leukocytes to the endothelium in the mucosal villi of small bowel mucosa following IR injury. PDTC also attenuated IR induced pulmonary tissue injury. These protective effects were clearly associated by the increased of HO-1 and abolished by the administration of ZnPP, a potent inhibitor of HO-1.

Our results also indicate that HO activity was increased in the IR group on comparison to the sham group. These results are similar to the findings of Patel *et al.* (Patel *et al.*, 2004). Previous studies also demonstrate HO expression is controlled at the level of gene transcription (Lee *et al.*, 1997;Ohlmann *et al.*, 2003). The probable explanation for the increase in IR group is that, hypoxia is well known to induce the expression of HO, ischaemia probably stimulates HO but mRNA synthesis is limited by the energy-depleted state of the cell. However, during reperfusion, cellular ATP pools are revived, thus enabling HO mRNA synthesis

The profound effects on the lungs following IR injury of the intestine may be induced by the activation of neutrophils following translocation of bacterial endotoxins. It is possible that the increased mucosal permeability following ischaemia allows passive

diffusion of endotoxin, which is a potent activator of neutrophils and could subsequently lead to remote multiple organ injury (Foulds *et al.*, 1997).

6.5 Conclusions

This chapter has shown that IR injury of the intestine induces rapid microcirculatory breakdown in addition to leukocyte adhesion in the mucosal layer with severe localized and systemic consequences. The administration of PDTC maintained a functioning mucosal microcirculation and markedly attenuated the IR injury. Thereby, PDTC may prove a prophylactic or therapeutic option to support the integrity of the gastrointestinal mucosa and to preserve or restore its barrier function. This may reduce translocation of toxins from the gastrointestinal lumen and consequently reduce the onset or severity of multiple organ failure.

Chapter 7 Haem oxygenase mediates microvascular protection in the late phase of ischaemia reperfusion injury of the intestine

7.1 Introduction

In the previous chapters it was shown that ischaemic preconditioning (IPC) and pharmacologic preconditioning with pyrrolidine dithiocarbamate (PDTC) protects the small bowel against ischaemia reperfusion (IR) injury of the intestine in the early phase (following 2hr) of reperfusion injury. This protection was afforded by the expression of haem oxygenase-1 (HO-1). In this chapter, the role of ischaemic and pharmacologic preconditioning in the late phase (following 24 hr) is examined.

The effects of IPC can be differentiated into two phases characterized by different time frames and mechanisms: (a) an early phase (early or classic preconditioning), that immediately follows the transient ischaemia and lasts 2–3 hr and (2) a late phase (late preconditioning), which begins 12–24 hr from the transient ischaemia and lasts for about 3–4 days(Mallick *et al.*, 2004).

7.2 Materials and Methods

Animal care and experimental protocols were performed in accordance with the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986. Male Sprague-Dawley rats weighing 250- 300g were used in this study. Rats were kept in temperature controlled environment with 12 hours light- dark cycle and allowed tap water and standard rat chow pellets ad libitum.

7.2.1 Operative Procedures

Animals were anesthetized using isoflurane (Baxter, Norfolk, UK) and allowed to breathe spontaneously via concentric mask connected to an oxygen regulator. The

animal's body temperature was maintained at 36-38 ^{o}C using a heating pad (Harvard apparatus Ltd., Kent, UK) and monitored with a rectal temperature probe. The arterial oxygen saturation (SaO_2) and heart rate (HR) were continuously monitored with a pulse oximeter (Ohmeda Biox 3740 pulse oximeter, Ohmeda Louisville, Colorado, USA). Laparotomy was carried out through a midline incision. The superior mesenteric artery (SMA) was identified and occluded with a non-traumatic vessel clamp to induce ischaemia. Reperfusion started when the clamp was released. Sham animals underwent the identical procedure but without placement of a clamp on the SMA. The incision was closed, 0.025 mL of subcutaneous carprofen was administrated, and the rats were allowed to recover with free access to food and water. 24 hours following ischaemia or at equivalent point in the sham group, animals were re-anesthetized for a terminal procedure. Rats were randomly allocated to 5 study groups (n=12/group). In each group RBC dynamics were studied on 6 animals and the other 6 animals had evaluation of the interactions of leukocyte to the endothelium.

7.2.2 Experimental Protocol

Group 1. Sham laparotomy, the SMA was identified with no vascular occlusion and animals received a single dose of 2mL/ kg of 0.9% normal saline subcutaneously.

Group 2. IR, the SMA was occluded for 30 min, followed by 24 hour period of reperfusion.

Group 3. IPC, 10 min of ischaemia followed by 10 min of reperfusion + as in Group II procedure.

Group 4. PDTC+IR. Animals received a single dose of 100 mg/kg of PDTC (Sigma Chemical Co, St Loius, Mo, USA) dissolved in 0.9% sodium chloride given subcutaneously 30 min before ischaemia. The time course and dose of PDTC used were based on previous studies by Liu *et al* (Liu *et al.*, 1999).

Group 5. (ZnPP+ IPC+ IR). ZnPP (Sigma Chemical Co, St Loius, Mo, USA) was dissolved in 0.2 mol/L sodium hydroxide and diluted in 0.9% sodium chloride. The dose of ZnPP was selected according to the experiment described by Tsuchihashi et al (Tsuchihashi *et al.*, 2003). Animals received a single dose of 1.5mg/Kg of ZnPP given subcutaneously 30 min before PDTC administration. The right jugular vein was cannulated with a polyethylene catheter (0.40-mm inner diameter, Portex, Kent, UK) for administering fluorochromes. The abdomen was re-opened through the previous incision. The ileum was identified and the mucosal surface was exposed in a segment of exteriorized ileum by making a 30 mm incision along the anti-mesenteric border using an electric microcautery. The animal's abdomen was covered with a plastic wrap (Saran wrap®, Dow Chemical, Michigan, USA) to prevent fluid evaporation.

7.2.3 In vivo Fluorescence Microscopy

The animals were placed on the stage of a Nikon custom built microscope (Nikon, Japan) as described previously.

Following 24 hrs of reperfusion, 6 animals received rhodamine 6G (0.2mL of 0.01%; Sigma UK) and other 6 received fluorescein isothiocyanate (FITC) (Sigma, UK) labelled RBC (0.5 mL) as a bolus injection from each group into the jugular vein.The dyes were prepared as described in the previous chapters. The following parameters were measured (a) mucosal perfusion index (MPI) and (b) RBC velocity on administration of FITC-labelled RBC

Rhodamine 6G evaluated the leukocyte-endothelial interactions in the mucosal villi (Dunn *et al.*, 2002).

The microscopy technique does not allow the entire mucosa to be kept under observation, hence two areas were pre-selected in each animal, at a distance from each other for detailed surveillance. Recordings were made from these areas every 10 min after the administration of the fluorochromes for a period of 30 minutes.

7.2.4 Mucosal Perfusion Index (MPI)

MPI was evaluated as described previously in chapter 4.3.4.

7.2.5 Mean Capillary Diameter (MCD)

The 40x lens was utilised to assess the MCD. Only villi showing perfusion were chosen for the assessment of this parameter.15 capillaries randomized across the capillary network were chosen and the mean was then determined by offline analysis by Lucia image grabber.

7.2.6 Capillary Red blood cell velocity

RBC velocity was measured as described previously in chapter 4.3.5.

7.2.7 Quantification of Leukocyte Adhesion

The number of leukocytes adherent to the endothelium of up to four separate villi in two separate areas was determined as described in chapter 4.3.6.

7.2.8 Haem Oxygenase Activity Assay

HO activity in ileal microsomal fractions was measured using a spectrophotometric assay of bilirubin production as described in the previous chapters.

7.2.9 Western blotting for HO-1

Ileal samples were also analyzed by the western immunoblotting technique as described in the previous chapters.

7.2.10 Biochemical Assays

Blood samples were taken from the inferior vena cava following 24 hr of reperfusion. The analysis of blood samples was performed as described previously in chapter 3.3.6.

7.2.11 Histological Investigations

At the end of the reperfusion period, samples of ileum and lungs were removed and analyzed for histological assessment as described previously.

7.2.12 Data Collection and statistical analysis

All data are expressed as mean ± standard error of mean (SEM) values. ANOVA and Bonferroni adjustment for multiple comparisons was used for statistical analysis. Unpaired Student's *t* test was used for comparison between two groups. $P < 0{\cdot}05$ was considered statistically significant.

7.3 Results

7.3.1 Mucosal Perfusion Index (MPI)

A homogenous microvascular perfusion with most of the villi being well perfused throughout the experiment was observed in sham operated animals. The mean MPI at the end of 24 hr reperfusion was 0.76 ± 0.04 (*Fig 7.1*).

In IR injury of the intestine, the mucosal perfusion was significantly decreased with many villi showing complete stasis. The mean MPI in the IR group at the end of 24 hr reperfusion was 0.36 ± 0.04 ($p<0.001$ vs. sham). In the IPC group, there was a significant increase in the mucosal perfusion compared to IR. The MPI in IPC at the end of 24 hr reperfusion was 0.68 ± 0.04 ($p<0.001$ vs. IR; $p>0.05$ vs. sham). PDTC led to an increase in MPI at the end of the 24 hr reperfusion and the mean value was 0.65± 0.04 ($p<0.001$ vs. IR). ZnPP led to a decrease in the MPI of 0.45± 0.08 ($p>0.05$ vs. IR; $p<0.001$ vs. PDTC and IPC).

7.4.2 Mean Capillary Diameter (MCD)

At the end of the reperfusion, the MCD in the sham operated animals was 7.43 ± 0.29. The MCD in IPC group was 6.49± 0.27 and in PDTC group it was 6.24± 0.36. IR and ZnPP led to an increase of MPI (11.75± 4.35 in IR and 8.81±2.42 in ZnPP), though this increase was not significant on comparison to IPC, sham and PDTC groups. ($p>0.05$) (*Fig. 7.2*)

7.4.3 Capillary Red blood cell Velocity

In the IR group RBC velocity decreased significantly compared with sham (0.17 ± 0.04 mm/s vs.0.36 ± 0.07 mm/s, $p<0.05$). IPC increased the RBC velocity to 0.38 ± 0.08 mm/s ($p<0.05$ vs. IR). The administration of PDTC improved the RBC velocity (0.34 ± 0.03 mm/s, $p<0.05$ vs. IR). ZnPP was associated with decreased RBC velocity of 0.11 ± 0.03 mm/ s ($p <0.05$ vs. PDTC) (*Fig.7.3*).

7.4.3 Leukocyte Adherence

In sham operated animals only a few leukocytes were found to be adherent within the endothelial lining of the mucosal villi (4.50 ± 1.56 cells/ length of villus). IR injury of the intestine induced a significant increase in the leukocyte adhesion in the endothelium (23.33 ± 2.27 cells/ length of villus; $p<0.01$ versus sham; *Fig.7.4*). It was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. IPC led to a significant decrease in the adhesion of the leukocytes to the endothelium (8.33 ± 2.45 cells/ length of villus $p<0.001$ vs. IR). ZnPP administration led to increase in the adhesion of leukocytes (16.83 ± 1.72 cells/ length of villus; $p<0.01$ vs. IPC), whereas PDTC administration significantly decreased the leukocyte adhesion in the endothelium (5.83± 1.16 cells/ length of villus; $p< 0.01$ vs. IR).

7.4.4 Haem Oxygenase activity and Western blot for HO-1

Fig. 7.5 depicts the mean ileal HO activity at the end of the reperfusion in all the five experimental groups in pico moles bilirubin/ mg/protein/hr. IR led to an increase of mean HO activity as compared to the sham group ($p<0.001$). IPC led to more than two-fold increase of HO activity (1719.25 ± 273.10) as compared to IR (708.01 ± 311.46) ($p<0.001$ vs. IR). The mean HO activity in PDTC group was 1462.91 ± 254.45 (p

<0.001 vs. IR). The mean HO activity in the ZnPP group was 173.56 ± 61.05 (p<0.001 vs. PDTC). *Fig 7.6* shows IPC induces the expression of HO-1 by western blotting.

7.4.5 Biochemical Assays

Fig 7.7 shows the LDH levels at the end of the reperfusion in all the five groups. The LDH value in sham group at the end of the reperfusion was 378 .33 ± 113.6 U/L. LDH was significantly reduced (P< 0.001) in IPC (587.5 ± 174.0 U/L) as compared to IR (1548.8 ± 301.2 U/L). PDTC reduced the LDH levels as compared to ZnPP. (428.0 ± 111.6 U/L vs. 1673.5 ± 480.4 (p<0.001).

7.4.6 Intestinal histology

After 24 hrs of reperfusion, disruption of mucosal villous architecture was observed in IR group. However, IPC and PDTC well preserved the mucosal pattern. ZnPP led to damage to the mucosal layer with necrosis *(Fig 7.8).*

7.4.7 Lung Histology

Following IR and with the administration of ZnPP, it was noted that the pulmonary alveoli were either collapsed or not distended to the same degree as in sham. (*Fig 7.9*) IR and ZnPP groups were associated with marked neutrophilic infiltration in the capillaries within the interstitium. IPC and PDTC attenuated injury to the pulmonary tissue with the alveoli being well aerated and few neutrophils.

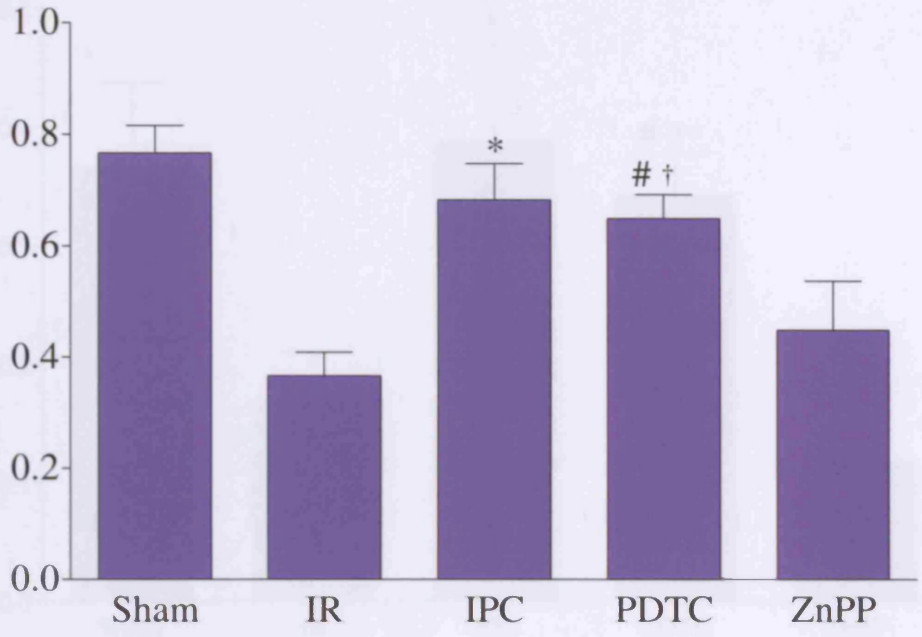


Figure 7.1. The mucosal perfusion index in all the five experimental groups.(* p<0.001 IPC vs. IR; # p<0.001 PDTC vs. IR; † p<0.001 PDTC vs. ZnPP)

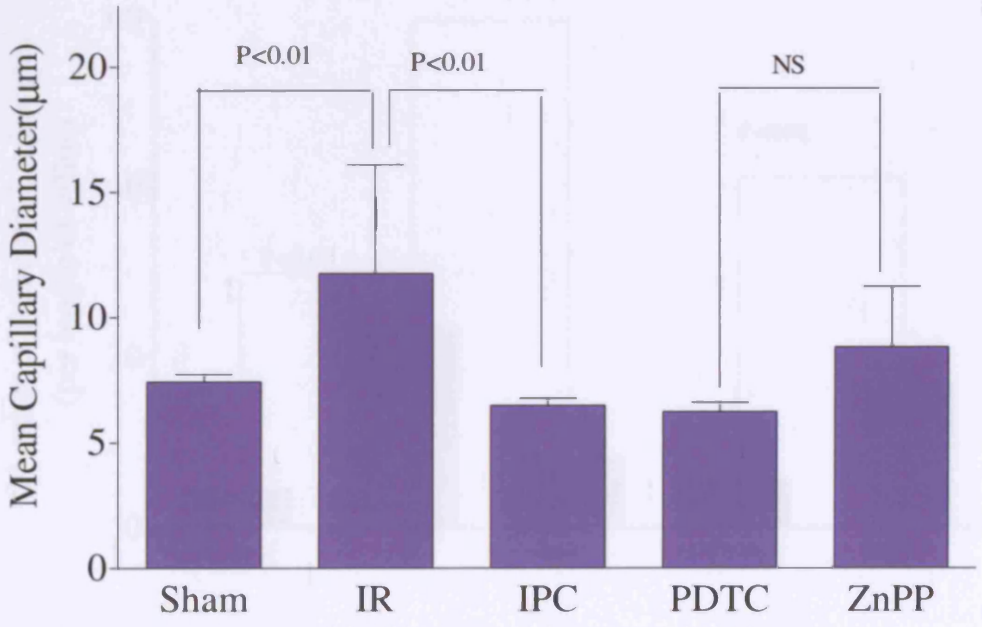


Figure 7.2. Mean capillary diameter in all the five experimental groups (NS- non-significant).

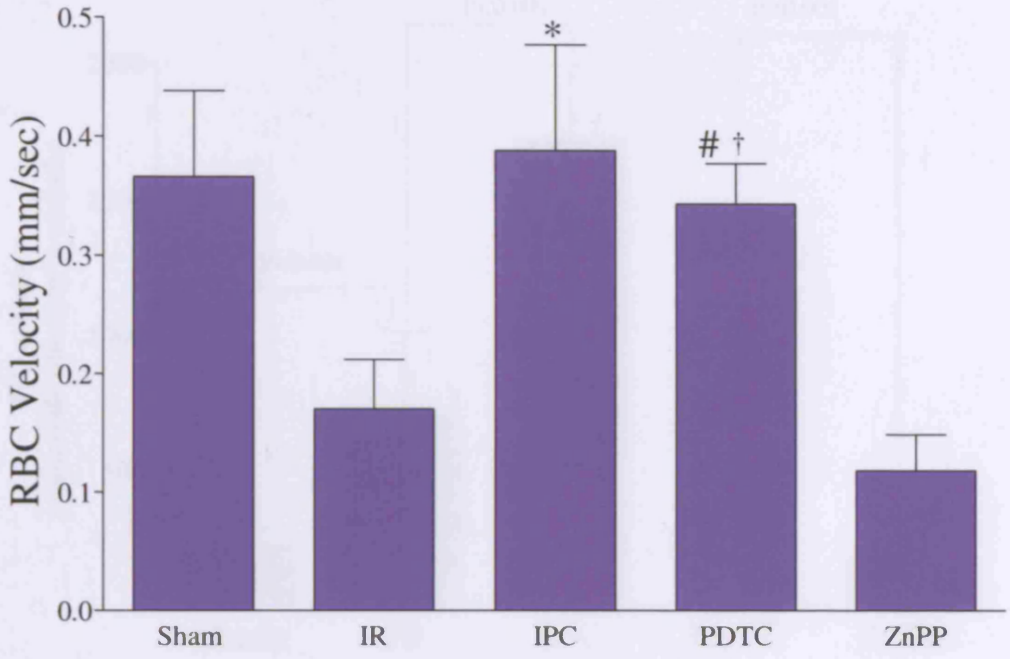


Figure 7.3 The red blood cell velocity in all the five experimental groups (*p<0.05 IPC vs. IR; # p<0.05 PDTC vs. IR; † PDTC vs. ZnPP)

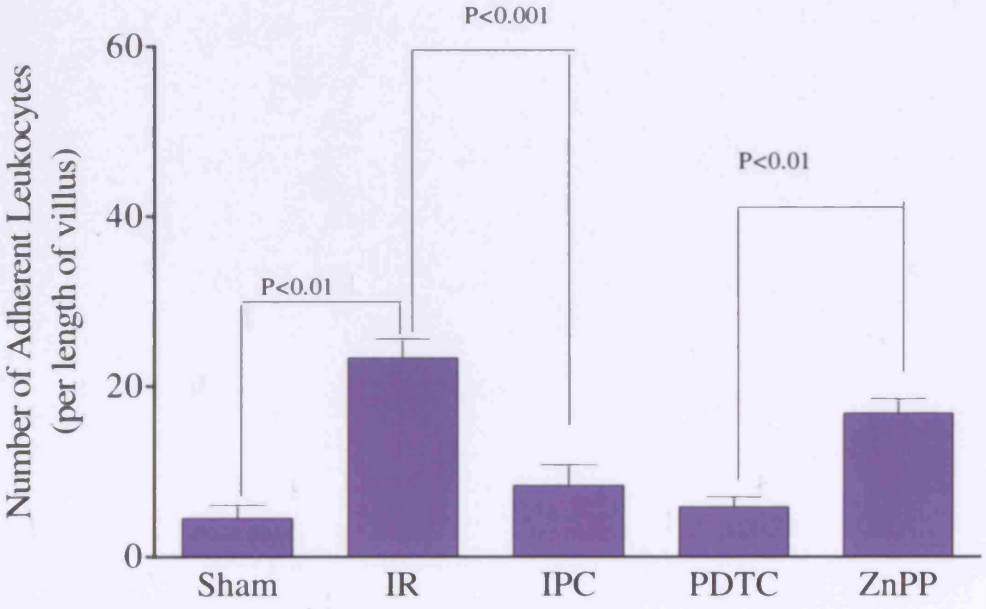


Figure 7.4 Leukocyte adhesion in all the five experimental groups.

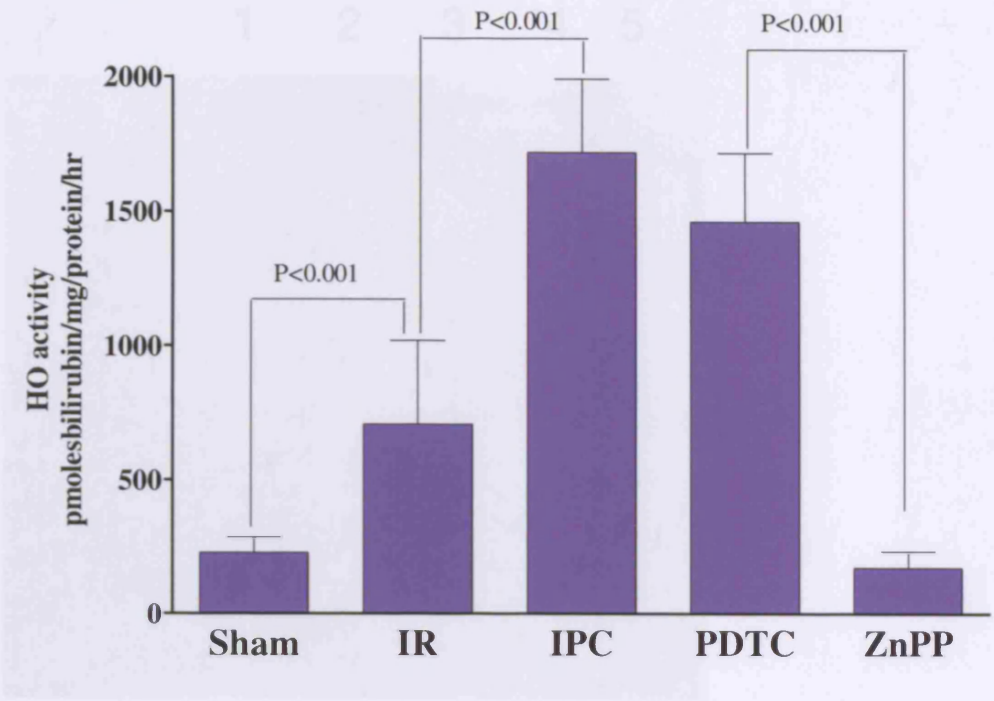
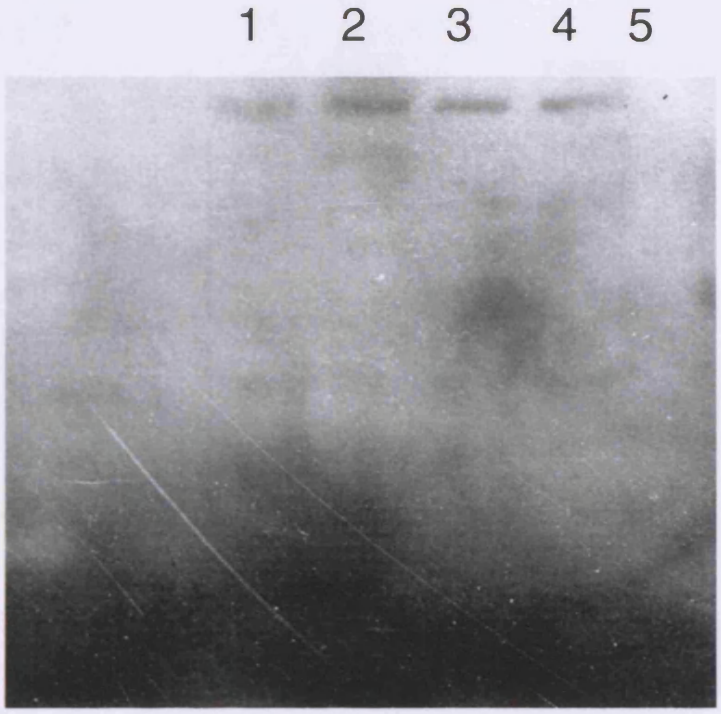


Figure 7.5. Mean HO activity in all the five experimental groups.



Lanes : 1. IR 2. IPC 3. PDTC 4. ZnPP 5. Sham

Figure 7.6 The expression of HO-1 at the end of 24 hrs of reperfusion detected by western blotting.

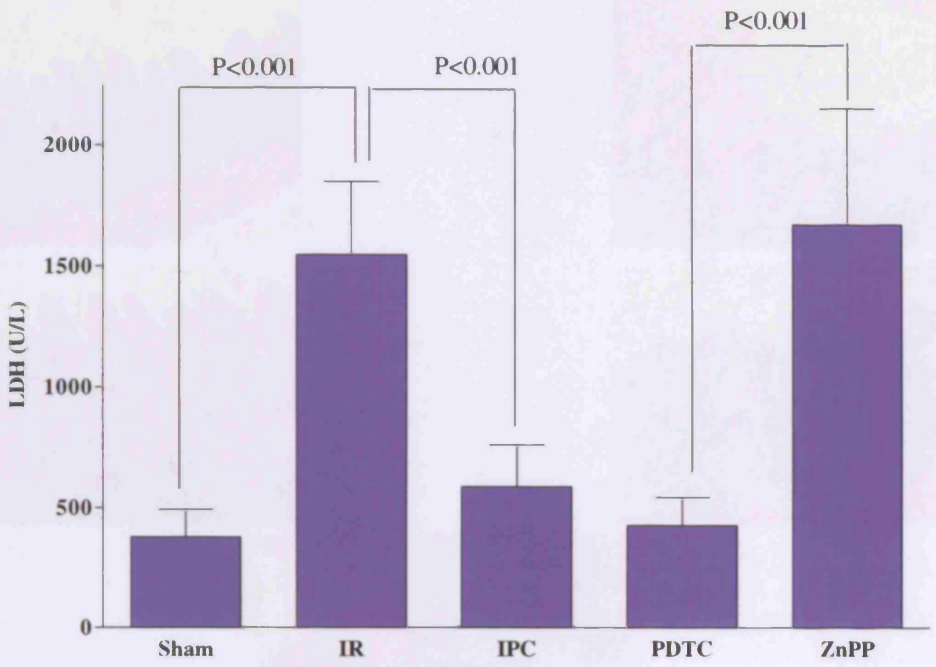


Figure 7.7 Mean serum LDH levels in the five experimental groups.

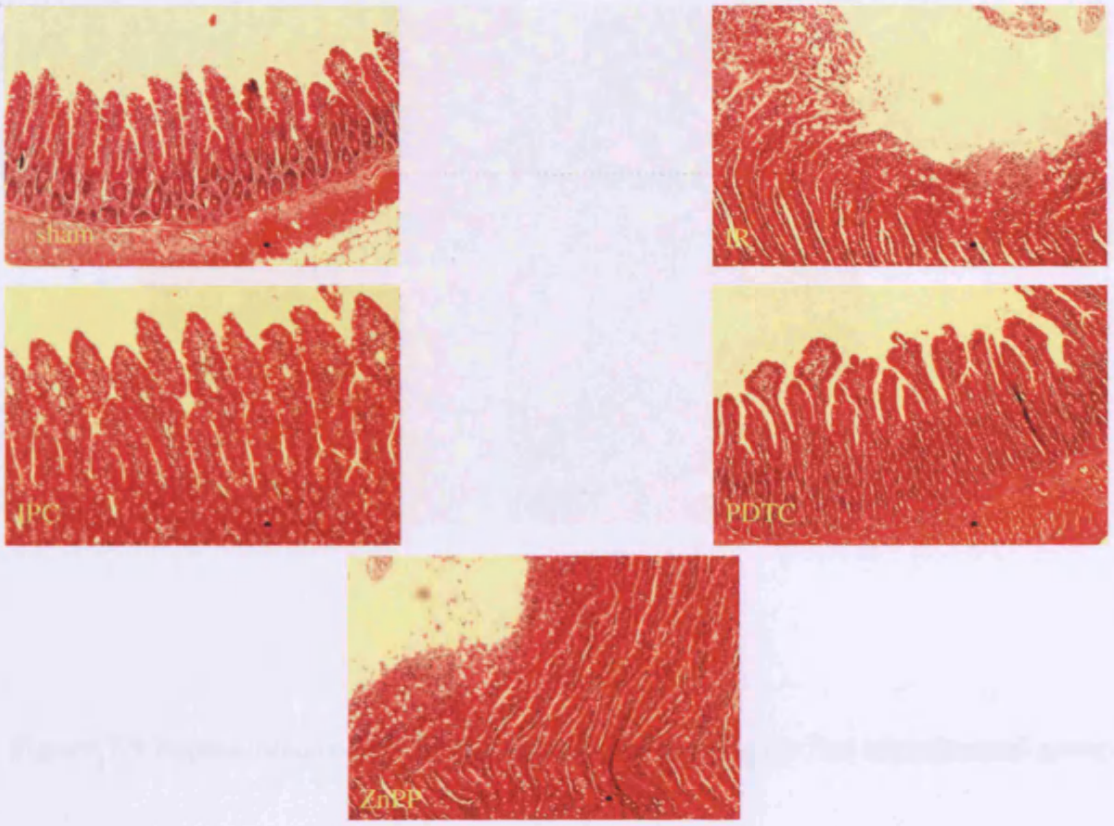


Figure 7.8 Representative histologic sections in the five experimental groups. IPC and PDTC preserved the mucosal villous pattern whereas IR and ZnPP led to mucosal necrosis.

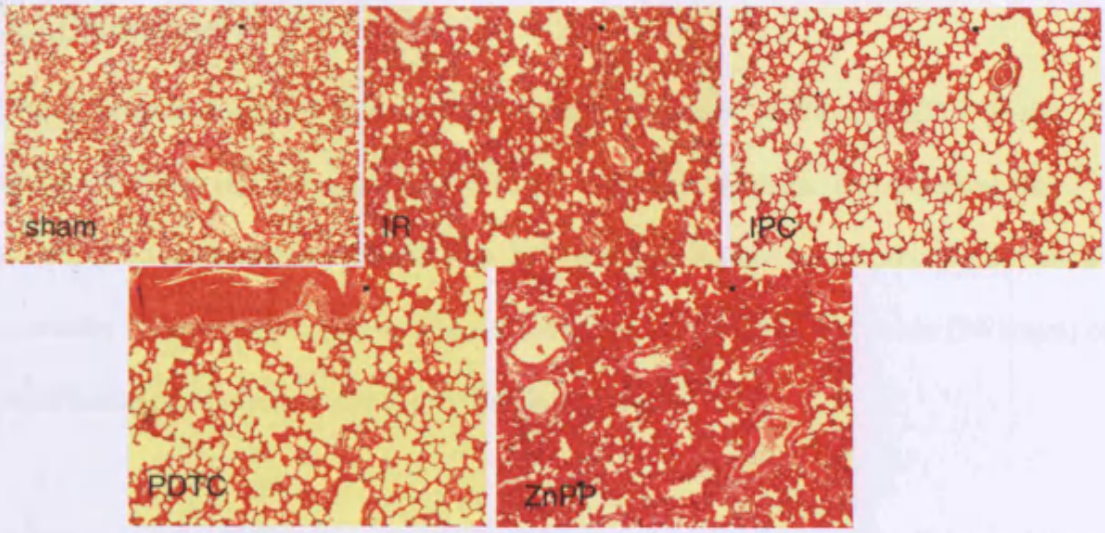


Figure 7.9 Representative lung histologic specimens from the five experimental groups.

7.5 Discussion

This chapter investigated the late effects of ischaemic preconditioning (IPC) on the intestinal microcirculation and how it relates to HO metabolism. The experimental model of 30 minutes of ischaemia with 24 hours reperfusion had no procedure-related mortality and was developed to evaluate intestinal injury in the late phase (24 hours) of reperfusion after substantial but non-lethal ischaemia.

In this study, to evaluate the effect of HO-1 on the intestinal microcirculation, additional groups had Pyrrolidine dithiocarbamate (PDTC) (HO-1 donor) and Zinc Protoporrphyrin (inhibitor of HO-1) administered before IR injury. The results of this chapter indicate that the beneficial effect of preconditioning could be related to an improvement in microcirculation and implicates HO-1 as a contributory mechanism. PDTC treatment protected against IR injury similar to preconditioning, whereas ZnPP administration aggravated microcirculatory impairment and cellular injury in preconditioned animals.

The results of the present study clearly demonstrate that PDTC treatment before IR resulted in significant improvement in intestinal microvascular perfusion and attenuation of leukocyte-endothelial interactions when compared with IR group. This suggests that mechanism modulated by IPC involve HO-1 production as shown by the increase of HO-1 expression. In contrast, the use of HO-1 synthesis inhibitor ZnPP with IPC in this study led to the derangement of the intestinal microcirculation as shown by decreased perfusion and increased leukocyte accumulation in the villi. Therefore HO-1 inhibition promotes leukocyte adhesion and has decreases intestinal microvascular perfusion. The results of this study also show that both IPC and PDTC treatment resulted in increased expression of HO-1, whereas administration of ZnPP resulted in

significant reductions in the expression of HO-1 indicating effective inhibition of HO-1. IR and ZnPP was associated with an increase of the capillary diameter which indicated the development of microvascular stasis.

This study has shown that IR injury of the intestine induces rapid microcirculatory breakdown in addition to leukocyte adhesion in the mucosal layer with severe pathologic consequences. IPC of the intestine can maintain a functioning mucosal microcirculation and markedly attenuate the IR injury. Thus, the data from this study emphasize the association of HO-1 with IR induced intestinal injury and suggest a positive relationship between IPC and the improvement in intestinal microcirculation and reduction in intestinal injury. The data from this study also suggest that HO-1 limits the progression of IR injury and inhibition of HO-1 synthesis blocks the preconditioning effect and exacerbates intestinal injury. These observations provide evidence that HO-1 is strongly associated with the preconditioning effect and may be an important factor in mediating the preconditioning response.

Chapter 8 Summary of the thesis

8.1 Summary

To our knowledge this is the first study investigating the tissue oxygenation and microdynamics of both red and white blood cells in the intestinal mucosa following IPC in the early and late phase of IR injury. Previously, Kubes *et al* showed that IPC of the feline intestine attenuated leukocyte adhesion in the mesenteric venules, changes in the mucosa were not addressed in this study (Kubes *et al.*, 1998). The mucosa of the small bowel is exceedingly sensitive to IR injury and even a short episode of ischaemia can induce substantial damage to the layer (Mallick *et al.*, 2004). The tissue damage in IR injury of the intestine is preferentially localized to the mucosa with the underlying submucosal layers virtually unaffected and hence it is vital to observe the changes in the mucosa. IR injury of the intestine was associated with decreased mucosal perfusion index, RBC velocity and increased leukocyte endothelial interactions in the mucosa. The increase in leukocyte-endothelial interactions was in concordance with the studies by Kalia et al (Kalia *et al.*, 2002). Our study has clearly demonstrated that IPC attenuates the microvascular disturbances by improving the mucosal perfusion index, RBC velocity and reduces the adhesion of leukocytes to the endothelium in the mucosal villi of small bowel mucosa following both early and late phases of IR injury. IPC also preserved the mucosal villous architecture. These protective effects were clearly accompanied by a marked expression of HO.

Recent studies have shown that induction of HO helps to ameliorate tissue injury or inflammatory changes in a variety of experimental models (Attuwaybi *et al.*, 2004;Tamion *et al.*, 2002). However these studies do not address the role of HO in the intestinal microcirculation.

Capillary no-reflow and leukocyte endothelial interaction hallmark IR injury of the intestine(Kaminski and Proctor, 1989). Following IR injury, many cell types may be damaged by oxygen free radicals with RBC one of the most vulnerable (Chien, 1982). Impaired antioxidant defenses or increased production of oxygen free radicals may disturb the critical balance and result in oxidative damage to the RBC (Claster *et al.*, 1984). Injury to RBC is manifested both morphologically and physiologically (Chien, 1982). Since damaged RBC tend to increase their deformity and aggregate, the RBC velocity may decrease as demonstrated in this study. In addition, RBC lysis may occur, both within the capillary lumen and in the extracellular space, thereby releasing a source of free haem which may further exacerbate IR injury.

The results of the present study clearly demonstrate that IPC induced maintenance of adequate capillary perfusion *in vivo*, and indicate that the protective effect of the IPC phenomenon may be mediated by the action of HO as shown by western blot analysis. In IR injury it was observed that adherent leukocytes were frequently plugging the capillaries leading to reduced or no flow within the villus microcirculation. Hayashi *et al.* demonstrated that overexpression of HO-1 in microvascular endothelial cells ameliorates oxidative injury and reduced the leukocyte endothelial interactions (Hayashi *et al.*, 1999).Hence, reduction of the leukocyte endothelial interaction by expression of HO-1 would reduce the plugging of the capillaries and thereby improve microvascular flow.

The profound effects on the lungs following IR injury of the intestine may be induced by the activation of neutrophils following translocation of bacterial endotoxins (Foulds *et al.*, 1997). It is possible that the increased mucosal permeability following ischaemia

allows passive diffusion of endotoxin, which is a potent activator of neutrophils and could subsequently lead to remote multiple organ injury.

This study has shown that IR injury of the intestine induces rapid microcirculatory breakdown in addition to leukocyte adhesion in the mucosal layer with severe pathologic consequences. IPC of the intestine can maintain a functioning mucosal microcirculation and markedly attenuate the IR injury. IPC also attenuated the IR induced pulmonary injury.

8.2 Methodological considerations

8.2.1 The Experimental model

The study of the cascade of phenomena related to ischaemia and reperfusion in the intestine is of particular interest due to the importance of ischaemia in abdominal and cardiac surgery, shock and small bowel transplantation. Ischaemia reperfusion injury of the intestine is inevitable following small bowel transplantation. The complexity of the intestinal microcirculation is also a major obstacle to reproduce and analyse pathophysiological mechanisms under experimental conditions.

The experimental model employed in this study offered the opportunity of assessment of both haemodynamic and microcirculatory parameters in the rat intestine, both under normal conditions and also when it is submitted to ischaemia and reperfusion.

In contrast with preservation of organs at low temperatures, normothermic ischaemia produces rapid injury to the intestine, with the possibility of in-vivo study without the systemic effect of transplantation itself, which represents a major disturbance for physiological investigations. Normothermic ischaemia of the intestine is, to some extent, a simulation of the circulatory and metabolic conditions of transplantation, being, nevertheless, a simple, reproducible and efficient experimental method, with low mortality and morbidity.

The model is ideal to study the relationship between post ischaemic intestinal blood flow and post ischaemic small bowel function. The experimental reproduction of these

phenomena at others levels is only possible with the application of sophisticated methods of isolated perfused organs or cell and tissue cultures.

The rat was selected as the experimental animal due to its resistance to surgical trauma and infection, the advantage of size and the intestinal microvascular anatomy which makes the technical procedure possible, compared with other laboratory animals.

The rat model of intestinal ischaemia and reperfusion is a useful procedure for the study of the local and systemic effects of ischaemia and reperfusion injury. This model offers a well defined volume of tissue that can be easily rendered ischaemic with little or no alteration of systemic haemodynarnics.

In this study, blood flow to the small bowel was interrupted by clamping of the superior mesenteric artery. Experimental data suggest that IR induced intestinal injury occurs in a biphasic manner; an early phase of injury that develops over the course of 2 h of reperfusion and a later progressive phase that develops at 6 to 24 hrs after reperfusion(Slavikova *et al.*, 1998). Hence 2hr of reperfusion was chosen as the early phase of reperfusion injury and 24 hr of reperfusion chosen as the late phase of reperfusion injury. The ischaemic times utilized in this study were selected on the basis of multiple experiments. This technique of intestinal ischaemia and reperfusion in the rat is easily performed and can always be reproduced; the initial ischaemic changes in the intestine are evident and leave no doubt of the proper positioning of the clamp. In addition, the procedure can be performed rapidly, and there was no procedure related mortality.

The next section addresses the application of various tests to assess the pathophysiology of small bowel ischaemia and reperfusion in this *in vivo* rat model. All the described tests are not necessarily applied to every experimental situation; sometimes for being redundant, or not applicable to the particular conditions of the experiment, in terms of ischaemic times utilized, periods of observation, or peculiarities of the drugs utilized.

8.2.2 Laser Doppler Flowmetry

The estimation of microcirculatory blood flow is of crucial interest for the analysis of the events that occur during organ ischaemia and reperfusion. Changes in the tissue blood flow of different organs during reperfusion have been described, and in case of the small bowel, there is now increasing evidence that impairment of the intestinal microcirculation is a major determinant of ischaemia reperfusion injury.

Laser Doppler Flowmetry is a reliable method for the continuous measurement of tissue blood flow. LDF has been validated as a method for measuring gastrointestinal microvascular blood flow in animal models(Feld *et al.*, 1982;Shepherd and Riedel, 1982)and humans(Thollander *et al.*, 1996). The LDF probe was placed on a fixed site on the serosa of the ileum and was held in place by a probe holder. Serosal blood flow has previously been shown to correlate well with mucosal flow(Corbett *et al.*, 2000). LDF data were collected continuously at sampling rate of 2Hz on computer.

This method does not measure the microcirculatory blood flow of the small bowel as a whole, which would be ideal, however, the location of the probe in a fixed point of the intestinal surface throughout the experiment allows the continuous record of the flow signal to be considered representative of the microcirculatory changes on that point

during the different stages of the procedure, using the same place as a control. A baseline recording of flow as a standard (100%) was used in each individual experiment; nevertheless, the absolute perfusion values in pre-ischaemic, anaesthetized rats are rather constant, being around 280 LDF Perfusion Units.

During the application of LDF during the experiments a few problems were encountered. The Laser Doppler monitor can produce artifact signals due to vibration or other movements of the fibreoptic probe itself and due to relative movements between the probe and the explored tissue. In our experimental model it was possible to obtain reasonably artifact-free readings with a good level of anaesthesia and leaving the probe loosely in contact with the ileal serosa (instead of firmly attached to a manipulator).

The unit measured with the LDF was expressed in arbitrary "Perfusion Units". This is because the backscattered light varies from one organ to another depending on factors including light absorption and red cell fraction of different tissues and it is not possible to translate the values into a unit of flux that can be utilized for different organs; nevertheless, when applied to the same organ the signal is reproducible within a narrow range of variation.

8.2.3 Near infrared spectroscopy

NIRS is a non-invasive technique to assess intestinal tissue oxygenation. A commercially available near infrared spectrophotometer that was developed for measurement of brain tissue oxygenation was used(Cope *et al.*, 1988). An algorithm designed specifically to measure intestinal tissue oxygenation was used.

During the application of NIRS during the experiments few problems were encountered. The interference by the effect of operating lights on the measurement requires dimming of the operating lights and covering the probes with lightproof black cloth. This could represent a minor difficulty in its clinical application. Variation in measurements were found with the reapplication of the probes even on the same site of measurement which may be caused by variation of the optical properties of the area under investigation which are tested and accounted for by the spectroscopy with the initial setting. This problem restricts the NIRS application as the probes must be applied and maintained in the same site without movement during the whole procedure, which may be impractical in clinical situation. Absolute quantification of the measurement will solve such problem.

The volume of blood changes during ischemia and reperfusion may affect the amount of light scattering. In theory, this may influence both NIRS as well as LDF measurements. As far as we know there are no reports of studies investigating the relationship between changes in light scattering and changes in blood volume in the rat small bowel in the context of ischaemia and reperfusion. In the brain for example, large changes in the cerebral energy state do not cause large changes in light scattering in hypoxic-ischaemic piglet brains. An assumption was made that the observed optical density changes are caused by altered chromophore absorbance and not light scattering. To determine absolute changes in chromophore concentration, the optical path length in the tissue must be known as a function of wavelength. The differential path length factor has been determined specifically for the small bowel by measuring the absorption coefficient as a function of wavelength. The differential path length factor of the small bowel is 2.7, and

this value was used to adjust the NIRS algorithm for calculating the changes in the chromophore concentration.

8.2.4 *In vivo* fluorescent microscopy

The term in vivo microscopy summarizes experimental approaches in which the microcirculation of organs is made accessible to direct observation. Although direct quantitative techniques are difficult to implement in the clinical scenario, nevertheless they provide advantages over indirect methods because the microcirculation does not respond uniformly to either physiological or pharmacological interventions. In vivo microscopy of the intestine allows assessment of the intestinal microvascular perfusion, the analysis of dynamic processes such as changes in diameters of blood vessels, interactions between leukocytes and endothelium.

For the purposes of in vivo microscopic assessments, a plane organ surface is necessary to provide clear and sharp images without inducing trauma to the tissue under the objective. The anaesthetized animals were placed on the stage of a Nikon custom built microscope (Nikon, Japan) with an integrated heating system where the temperature was maintained at 37°C. The whole set up was placed on a pneumatic vibration isolation workstation (Newport, USA) to minimise vibration. The mucosal surface was exposed in a segment of exteriorized ileum by making a 30 mm incision along the anti-mesenteric border using an electric microcautery. The small bowel was placed on to a specially designed plastic stage and a cover slip was then sited on to the mucosa. This procedure was easily tolerated by the animals and allowed the tissue to be exteriorized with a minimum of trauma. This method of exteriorization of the ileum also eliminated the respiratory movements in the tissue. The mucosal surface was kept moist with isotonic saline. During the experiments, the animal's abdomen was covered with a

plastic wrap (Saran wrap®, Dow Chemical, Michigan, USA) to prevent fluid evaporation. The loops of small bowel must be carefully handled, as mechanical trauma induces disturbances in microcirculation. Microhaemorrhages falsify the results and can make clear observation impossible. In order to obtain clear images, it is necessary to match the concentration of fluorescent dyes with the filter systems, the light intensity and the magnification.

Evaluation of microcirculatory parameters has to be performed in conjunction with findings from the macrocirculation. Arterial blood pressure and heart rate were measured during the experiments in order to fulfil this requirement.

We measured the following parameters using the fluorescent dyes: mucosal perfusion, capillary diameter, red blood cell velocity and leukocyte-endothelial interactions. Evaluation of the microscopic images depends on subjective viewpoints rather than absolute values.

8.2.5 Serum Lactate dehydrogenase and liver enzymes

Previous studies have shown that the estimation of serum lactate dehydrogenase (LDH) is a very sensitive marker of intestinal ischaemia(Caglayan *et al.*, 2002). Blood levels of intracellular enzymes are a way of estimating tissue damage: tissue or organ specific enzyme levels provide valuable information about related tissues. LDH released from the damaged intestine during ischaemia enters the circulation during reperfusion and their blood levels increase. The addition of reperfusion injury and damage to distant organs will further increase the blood level enzymes.

ALT and AST are indicators of major alterations of liver integrity, and were utilized in this study as a marker of hepatocellular injury.

8.2.6 Haem Oxygenase studies

Haem oxygenase-1 (HO-1) is a ubiquitous and redox-sensitive inducible stress protein that degrades haem to carbon monoxide (CO), iron and biliverdin. In this study HO estimations were used as marker of HO production and to affirm the response to pharmacological manipulation with Pyrrolidine dithiocarbamate and Zinc Protoporphyrin. HO was measured by the bilirubin generation method as described by Balla *et al.* (Balla *et al.*, 1992) and is well established and of proven efficacy.

Polyclonal antibodies against the specific HO isoforms are commercially available and can identify the isoforms on tissues sections and these were used for western blotting in this study. A limitation of molecular biology techniques is that they only indicate the presence of protein and as such may indicate synthesis of new protein but these techniques do not give an indication of protein phosphorylation and therefore enzymatic activity. The other option is to use enzyme phosphorylation assays, but these are indirect measurements and again do not help in establishing a direct link between protein and enzymatic, In theory, a combination of specific HO isoform genetic knockout model and molecular biology techniques would give a better understanding of HO-1 activity in a given setting.

8.2.7 Histology

Light microscopy examination allows excellent appraisal of degree of tissue injury. In the ileum the end points of enterocyte necrosis, endothelial injury and neutrophil infiltration were chosen as histologic markers, realizing that more detailed ultrastructural studies may be useful either to confirm or to explain histological findings in some cases. In this study, clear differences were found between groups, and histology was of great help to understand the nature of ischaemic injury.

8.3 Overall conclusions arising from the thesis and future plans

1. Ischaemic preconditioning was associated with an improvement in the intestinal microvascular perfusion, intestinal extra and intracellular oxygenation and portal venous blood flow.
2. Ischaemic Preconditioning led to an improvement of red blood cell velocity (i.e. blood flow) and decreased the leukocyte- endothelial interactions. This protective effect of ischaemic preconditioning was clearly associated with an increased expression in haem oxygenase in the early phase of ischaemic preconditioning.

3. The administration of HO-1 donor Pyrrolidine dithiocarbamate produced results similar to ischaemic preconditioning, i.e. improvement in the intestinal microvascular perfusion, intestinal extra and intracellular oxygenation, portal venous blood flow, improved red blood cell velocity and attenuated the leukocyte-endothelial interactions. Pyrrolidine dithiocarbamate administration was associated with increased expression of HO-1, whereas the administration of Zinc Protoporphyrin (HO-1 inhibitor) abolished this protective effect.

4. Ischaemic Preconditioning led to a dramatic improvement of red blood cell velocity (i.e. blood flow) and decreased the leukocyte- endothelial interactions as compared to ischaemia reperfusion. This protective effect of ischaemic preconditioning was clearly associated with an increased expression in HO-1.

5. The administration of HO-1 donor Pyrrolidine dithiocarbamate produced results similar to ischaemic preconditioning. Pyrrolidine dithiocarbamate administration was associated with increased expression of HO-1, whereas the administration of Zinc Protoporphyrin (HO-1 inhibitor) abolished this protective effect.

6. Ischaemia reperfusion injury of the intestine led to damage of the alveolar endothelium, whereas ischaemic preconditioning attenuated the pulmonary injury and therefore limited distant organ injury.

The above conclusions support the hypothesis presented in this study and strongly suggest a role for HO-1 in the protection conferred by IPC in early and late phase of ischaemia reperfusion induced intestinal injury. The potential clinical application of the IPC effect is during small bowel transplantation. Although the technique of IPC is simple and easily applicable there would be concerns such as the increased operative time (due to period of time involving brief ischaemia and reperfusion) which may not be tolerated well in many operating theatres. Also other concerns such as, what is the ideal preconditioning time?, would IPC influence other diseased states of the intestine (such as post chemotherapy, shock states), these questions largely remained answered since critical ischaemia times for the intestine particularly with the trauma of surgery and disease are not known. Therefore the potential clinical application of IPC is in the form of targeted drug intervention. Although it may be debatable whether

preconditioning will ever fulfill its clinical expectations, the hope that a new therapeutic modality may emerge from this fascinating phenomenon has given great impetus to the search for its underlying mechanism. Clearly, identifying the mechanism of IPC will allow development of pharmaceutical agents that conduct the IPC response. Such agents will vastly assist preservation of intestine for transplantation. The overall conclusions of this study suggest a central role for HO-1 in the IPC cascade. Further mechanistically descriptive studies would allow the development of therapeutic regimens involving HO-1 e.g., regime for HO donors administration prior to small bowel transplantation.

This study has shown that HO-1 is upregulated during the IPC effect. There are few questions which remain answered because some were not within the scope of this study. These questions could open avenues for future studies. Where was HO-1 expressed in the ileum? It would be useful to localize the expression of HO-1 in the small intestine by means of immunohistochemistry. It would also be useful to find out the expression of HO-1 at different time points starting from basal expression, before and after ischaemia and during reperfusion.

Finally, although current research on the mechanisms of preconditioning seems to diverge more and more, it is possible that all these mechanisms converge into an as yet unidentified final common pathway. This conclusion is based not so much on the negative studies and observations discussed above but on the belief that a powerful adaptive phenomenon that is induced so easily and reproducibly in so many models, laboratories, and species is almost certain to be mediated by a universal mechanism. The data from this study suggests that haem oxygenase is the key factor in small bowel ischaemic preconditioning.

Future Plans

The temporal expression of haem oxygenase-1 (HO-1) with varying time points of ischaemia and reperfusion will be useful if finding out at what time timepoint HO-1 is expressed. It will also be informative to find out the exact localization of HO-1 in the ileum (which cell expresses HO-1) by means of immunohistochemistry.

These experiments were performed in warm IR injury model, this has limitations in small bowel transplantation model. The experimental model of cold IR injury with preservation will mimic small bowel transplantation.

Reference List

Ahmadinejad M, Rex M, Sutton R H, Pollitt C C and Cribb B (1996) The Effects of Allopurinol on the Ultrastructure of Ischaemic and Reperfused Large Intestine of Sheep. *Aust Vet J* **74**: pp 135-139.

Ahn H, Lindhagen J, Nilsson G E, Salerud E G, Jodal M and Lundgren O (1985) Evaluation of Laser Doppler Flowmetry in the Assessment of Intestinal Blood Flow in Cat. *Gastroenterology* **88**: pp 951-957.

Akcakaya A, Alimoglu O, Sahin M and Abbasoglu S D (2002) Ischemia-Reperfusion Injury Following Superior Mesenteric Artery Occlusion and Strangulation Obstruction. *J Surg Res* **108**: pp 39-43.

Aksoyek S, Cinel I, Avlan D, Cinel L, Ozturk C, Gurbuz P, Nayci A and Oral U (2002) Intestinal Ischemic Preconditioning Protects the Intestine and Reduces Bacterial Translocation. *Shock* **18**: pp 476-480.

Albrecht EW, Stegeman C A, Heeringa P, Henning R H and van Goor H (2003) Protective Role of Endothelial Nitric Oxide Synthase. *J Pathol* **199**: pp 8-17.

Anadol AZ, Bayram O, Dursun A and Ercan S (1998) Role of Endogenous Endothelin Peptides in Intestinal Ischemia-Reperfusion Injury in Rats. *Prostaglandins Leukot Essent Fatty Acids* **59**: pp 279-283.

Anaya-Prado R, Toledo-Pereyra L H, Lentsch A B and Ward P A (2002) Ischemia/Reperfusion Injury. *J Surg Res* **105**: pp 248-258.

Andrasi TB, Kekesi V, Blazovics A, Dobi I, Szabo G and Juhasz-Nagy A (2002) ET(A) Receptor Blockade Protects the Small Intestine Against Ischaemia/Reperfusion Injury in Dogs Via an Enhancement of Antioxidant Defences. *Clin Sci (Lond)* **103 Suppl 48**: pp 59S-63S.

Aoki N, Johnson G, III and Lefer A M (1990) Beneficial Effects of Two Forms of NO Administration in Feline Splanchnic Artery Occlusion Shock. *Am J Physiol* **258**: pp G275-G281.

Arumugam TV, Shiels I A, Margolin S B, Taylor S M and Brown L (2002a) Pirfenidone Attenuates Ischaemia-Reperfusion Injury in the Rat Small Intestine. *Clin Exp Pharmacol Physiol* **29**: pp 996-1000.

Arumugam TV, Shiels I A, Woodruff T M, Reid R C, Fairlie D P and Taylor S M (2002b) Protective Effect of a New C5a Receptor Antagonist Against Ischemia-Reperfusion Injury in the Rat Small Intestine. *J Surg Res* **103**: pp 260-267.

Askanazi J, Carpentier Y A, Michelsen C B, Elwyn D H, Furst P, Kantrowitz L R, Gump F E and Kinney J M (1980) Muscle and Plasma Amino Acids Following Injury. Influence of Intercurrent Infection. *Ann Surg* **192**: pp 78-85.

Attuwaybi BO, Kozar R A, Moore-Olufemi S D, Sato N, Hassoun H T, Weisbrodt N W and Moore F A (2004) Heme Oxygenase-1 Induction by Hemin Protects Against Gut Ischemia/Reperfusion Injury. *J Surg Res* **118**: pp 53-57.

Austen Jr WG, Kobzik L, Carroll M C, Hechtman H B and Moore Jr F D (2003) The Role of Complement and Natural Antibody in Intestinal Ischemia-Reperfusion Injury. *Int J Immunopathol Pharmacol* **16**: pp 1-8.

Bagge U, Amundson B and Lauritzen C (1980) White Blood Cell Deformability and Plugging of Skeletal Muscle Capillaries in Hemorrhagic Shock. *Acta Physiol Scand* **108**: pp 159-163.

Balla G, Jacob H S, Balla J, Rosenberg M, Nath K, Apple F, Eaton J W and Vercellotti G M (1992) Ferritin: a Cytoprotective Antioxidant Strategem of Endothelium. *J Biol Chem* **267**: pp 18148-18153.

Balogh N, Krausz F, Levai P, Ribiczeyne P S, Vajdovich P and Gaal T (2002) Effect of Deferoxamine and L-Arginine Treatment on Lipid Peroxidation in an Intestinal Ischaemia-Reperfusion Model in Rats. *Acta Vet Hung* **50**: pp 343-356.

Barnes PJ (1997) Nuclear Factor-Kappa B. *Int J Biochem Cell Biol* **29**: pp 867-870.

Bauer M and Bauer I (2002) Heme Oxygenase-1: Redox Regulation and Role in the Hepatic Response to Oxidative Stress. *Antioxid Redox Signal* **4**: pp 749-758.

Berg RD (1999) Bacterial Translocation From the Gastrointestinal Tract. *Adv Exp Med Biol* **473**: pp 11-30.

Berlanga J, Prats P, Remirez D, Gonzalez R, Lopez-Saura P, Aguiar J, Ojeda M, Boyle J J, Fitzgerald A J and Playford R J (2002) Prophylactic Use of Epidermal Growth Factor Reduces Ischemia/Reperfusion Intestinal Damage. *Am J Pathol* **161**: pp 373-379.

Blackwell TS and Christman J W (1997) The Role of Nuclear Factor-Kappa B in Cytokine Gene Regulation. *Am J Respir Cell Mol Biol* **17**: pp 3-9.

Bohlen HG (1998) Integration of Intestinal Structure, Function, and Microvascular Regulation. *Microcirculation* **5**: pp 27-37.

Bolli R (2000) The Late Phase of Preconditioning. *Circ Res* **87**: pp 972-983.

Born GV, Melling A and Whitelaw J H (1978) Laser Doppler Microscope for Blood Velocity Measurements. *Biorheology* **15**: pp 163-172.

Bowles MJ, Pockley A G and Wood R F (2000) Effect of Anti-LFA-1 Monoclonal Antibody on Rat Small Bowel Allograft Survival and Circulating Leukocyte Populations. *Transpl Immunol* **8**: pp 75-80.

Brune B and Ullrich V (1987) Inhibition of Platelet Aggregation by Carbon Monoxide Is Mediated by Activation of Guanylate Cyclase. *Mol Pharmacol* **32**: pp 497-504.

Buyukgebiz O, Aktan A O, Yegen C, Yalcin A S, Haklar G, Yalin R and Ercan Z S (1994) Captopril Increases Endothelin Serum Concentrations and Preserves Intestinal Mucosa After Mesenteric Ischemia-Reperfusion Injury. *Res Exp Med (Berl)* **194**: pp 339-348.

Caglayan F, Caglayan O, Gunel E, Elcuman Y and Cakmak M (2002) Intestinal Ischemia-Reperfusion and Plasma Enzyme Levels. *Pediatr Surg Int* **18**: pp 255-257.

Carden DL and Granger D N (2000) Pathophysiology of Ischaemia-Reperfusion Injury. *J Pathol* **190**: pp 255-266.

Carden DL, Smith J K and Korthuis R J (1990) Neutrophil-Mediated Microvascular Dysfunction in Postischemic Canine Skeletal Muscle. Role of Granulocyte Adherence. *Circ Res* **66**: pp 1436-1444.

Carmody IC, Meng L, Shen X D, Anselmo D, Gao F, Ke B, Ma J P, Kupiec-Weglinski J W, McDiarmid S V, Busuttil R W, Shaw G and Farmer D G (2004) P-Selectin Knockout Mice Have Improved Outcomes With Both Warm Ischemia and Small Bowel Transplantation. *Transplant Proc* **36**: pp 263-264.

Ceppa EP, Fuh K C and Bulkley G B (2003) Mesenteric Hemodynamic Response to Circulatory Shock. *Curr Opin Crit Care* **9**: pp 127-132.

Ceran C, Sonmez K, Turkyllmaz Z, Demirogullarl B, Dursun A, Duzgun E, Basaklar A C and Kale N (2001) Effect of Bilirubin in Ischemia/Reperfusion Injury on Rat Small Intestine. *J Pediatr Surg* **36**: pp 1764-1767.

Chance B, Wang N G, Maris M, Nioka S and Sevick E (1992) Quantitation of Tissue Optical Characteristics and Hemoglobin Desaturation by Time- and Frequency-Resolved Multi-Wavelength Spectrophotometry. *Adv Exp Med Biol* **317**: pp 297-304.

Chen Y, Hung W T, Chen S M and Tseng S H (2002) Suppression of Elevated Plasma Interleukin-8 Levels Due to Total Ischemia and Reperfusion of the Small Intestine by Luminal Perfusion With Fetal Bovine Serum. *Pediatr Surg Int* **18**: pp 107-109.

Chien S (1982) Rheology in the Microcirculation in Normal and Low Flow States. *Adv Shock Res* **8**: pp 71-80.

Chiu CJ, McArdle A H, Brown R, Scott H J and Gurd F N (1970) Intestinal Mucosal Lesion in Low-Flow States. I. A Morphological, Hemodynamic, and Metabolic Reappraisal. *Arch Surg* **101**: pp 478-483.

Choi AM and Alam J (1996) Heme Oxygenase-1: Function, Regulation, and Implication of a Novel Stress-Inducible Protein in Oxidant-Induced Lung Injury. *Am J Respir Cell Mol Biol* **15**: pp 9-19.

Cicalese L, Lee K, Schraut W, Watkins S, Borle A and Stanko R (1996) Pyruvate Prevents Ischemia-Reperfusion Mucosal Injury of Rat Small Intestine. *Am J Surg* **171**: pp 97-100.

Cicalese L, Sileri P, Green M, Abu-Elmagd K, Kocoshis S and Reyes J (2001) Bacterial Translocation in Clinical Intestinal Transplantation. *Transplantation* **71**: pp 1414-1417.

Cinel I, Avlan D, Cinel L, Polat G, Atici S, Mavioglu I, Serinol H, Aksoyek S and Oral U (2003) Ischemic Preconditioning Reduces Intestinal Epithelial Apoptosis in Rats. *Shock* **19**: pp 588-592.

Cinelli AR (1998) Flexible Method to Obtain High Sensitivity, Low-Cost CCD Cameras for Video Microscopy. *J Neurosci Methods* **85**: pp 33-43.

Claster S, Chiu D T, Quintanilha A and Lubin B (1984) Neutrophils Mediate Lipid Peroxidation in Human Red Cells. *Blood* **64**: pp 1079-1084.

Clavien PA, Selzner M, Rudiger H A, Graf R, Kadry Z, Rousson V and Jochum W (2003) A Prospective Randomized Study in 100 Consecutive Patients Undergoing Major Liver Resection With Versus Without Ischemic Preconditioning. *Ann Surg* **238**: pp 843-850.

Clozel M, Gray G A, Breu V, Loffler B M and Osterwalder R (1992) The Endothelin ETB Receptor Mediates Both Vasodilation and Vasoconstriction in Vivo. *Biochem Biophys Res Commun* **186**: pp 867-873.

Cohen MV, Baines C P and Downey J M (2000) Ischemic Preconditioning: From Adenosine Receptor of KATP Channel. *Annu Rev Physiol* **62**: pp 79-109.

Coito AJ, Buelow R, Shen X D, Amersi F, Moore C, Volk H D, Busuttil R W and Kupiec-Weglinski J W (2002) Heme Oxygenase-1 Gene Transfer Inhibits Inducible Nitric Oxide Synthase Expression and Protects Genetically Fat Zucker Rat Livers From Ischemia-Reperfusion Injury. *Transplantation* **74**: pp 96-102.

Collard CD and Gelman S (2001) Pathophysiology, Clinical Manifestations, and Prevention of Ischemia-Reperfusion Injury. *Anesthesiology* **94**: pp 1133-1138.

Collins T, Read M A, Neish A S, Whitley M Z, Thanos D and Maniatis T (1995) Transcriptional Regulation of Endothelial Cell Adhesion Molecules: NF-Kappa B and Cytokine-Inducible Enhancers. *FASEB J* **9**: pp 899-909.

Cooper D, Russell J, Chitman K D, Williams M C, Wolf R E and Granger D N (2004) Leukocyte Dependence of Platelet Adhesion in Postcapillary Venules. *Am J Physiol Heart Circ Physiol* **286**: pp H1895-H1900.

Cope M, Delpy D T, Reynolds E O, Wray S, Wyatt J and van der Z P (1988) Methods of Quantitating Cerebral Near Infrared Spectroscopy Data. *Adv Exp Med Biol* **222**: pp 183-189.

Corbett EJ, Barry B N, Pollard S G, Lodge J P and Bellamy M C (2000) Laser Doppler Flowmetry Is Useful in the Clinical Management of Small Bowel Transplantation. The Liver Transplant Group. *Gut* **47**: pp 580-583.

Cuzzocrea S, Mazzon E, Costantino G, Serraino I, De Sarro A and Caputi A P (2000) Effects of N-Acetylcysteine in a Rat Model of Ischemia and Reperfusion Injury. *Cardiovasc Res* **47**: pp 537-548.

Cuzzocrea S, Mazzon E, Dugo L, Caputi A P, Aston K, Riley D P and Salvemini D (2001) Protective Effects of a New Stable, Highly Active SOD Mimetic, M40401 in Splanchnic Artery Occlusion and Reperfusion. *Br J Pharmacol* **132**: pp 19-29.

Cuzzocrea S, Tailor A, Zingarelli B, Salzman A L, Flower R J, Szabo C and Perretti M (1997) Lipocortin 1 Protects Against Splanchnic Artery Occlusion and Reperfusion Injury by Affecting Neutrophil Migration. *J Immunol* **159**: pp 5089-5097.

Cuzzocrea S, Zingarelli B and Caputi A P (1998) Role of Constitutive Nitric Oxide Synthase and Peroxynitrite Production in a Rat Model of Splanchnic Artery Occlusion Shock. *Life Sci* **63**: pp 789-799.

Damber JE, Lindahl O, Selstam G and Tenland T (1982) Testicular Blood Flow Measured With a Laser Doppler Flowmeter: Acute Effects of Catecholamines. *Acta Physiol Scand* **115**: pp 209-215.

Davis JM, Gute D C, Jones S, Krsmanovic A and Korthuis R J (1999) Ischemic Preconditioning Prevents Postischemic P-Selectin Expression in the Rat Small Intestine. *Am J Physiol* **277**: pp H2476-H2481.

De Blasi RA, Almenrader N and Ferrari M (1997) Brain Oxygenation Monitoring During Cardiopulmonary Bypass by Near Infrared Spectroscopy. *Adv Exp Med Biol* **413**: pp 97-104.

Deitch EA (1990) The Role of Intestinal Barrier Failure and Bacterial Translocation in the Development of Systemic Infection and Multiple Organ Failure. *Arch Surg* **125**: pp 403-404.

Delpy DT, Cope M, van der Z P, Arridge S, Wray S and Wyatt J (1988) Estimation of Optical Pathlength Through Tissue From Direct Time of Flight Measurement. *Phys Med Biol* **33**: pp 1433-1442.

Dowdall JF, Winter D C and Bouchier-Hayes D J (2002) Inosine Modulates Gut Barrier Dysfunction and End Organ Damage in a Model of Ischemia-Reperfusion Injury. *J Surg Res* **108**: pp 61-68.

Droy-Lefaix MT, Drouet Y, Geraud G, Hosford D and Braquet P (1991) Superoxide Dismutase (SOD) and the PAF-Antagonist (BN 52021) Reduce Small Intestinal Damage Induced by Ischemia-Reperfusion. *Free Radic Res Commun* **12-13 Pt 2**: pp 725-735.

Du ZY, Hicks M, Winlaw D, Spratt P and Macdonald P (1996) Ischemic Preconditioning Enhances Donor Lung Preservation in the Rat. *J Heart Lung Transplant* **15**: pp 1258-1267.

Dun Y, Hao Y B, Wu Y X, Zhang Y and Zhao R R (2001) Protective Effects of Nitroglycerin-Induced Preconditioning Mediated by Calcitonin Gene-Related Peptide in Rat Small Intestine. *Eur J Pharmacol* **430**: pp 317-324.

Dunn KW, Sandoval R M, Kelly K J, Dagher P C, Tanner G A, Atkinson S J, Bacallao R L and Molitoris B A (2002) Functional Studies of the Kidney of Living Animals Using Multicolor Two-Photon Microscopy. *Am J Physiol Cell Physiol* **283**: pp C905-C916.

Einav S, Berman H J, Fuhro R L, DiGiovanni P R, Fine S and Fridman J D (1975) Measurement of Velocity Profiles of Red Blood Cells in the Microcirculation by Laser Doppler Anemometry (LDA). *Biorheology* **12**: pp 207-210.

El Desoky AE, Delpy D T, Davidson B R and Seifalian A M (2001) Assessment of Hepatic Ischaemia Reperfusion Injury by Measuring Intracellular Tissue Oxygenation Using Near Infrared Spectroscopy. *Liver* **21**: pp 37-44.

El Desoky AE, Seifalian A, Cope M, Delpy D and Davidson B (1999) Changes in Tissue Oxygenation of the Porcine Liver Measured by Near-Infrared Spectroscopy. *Liver Transpl Surg* **5**: pp 219-226.

Elwell CE, Cope M, Edwards A D, Wyatt J S, Delpy D T and Reynolds E O (1994) Quantification of Adult Cerebral Hemodynamics by Near-Infrared Spectroscopy. *J Appl Physiol* **77**: pp 2753-2760.

Farmer DG, Amersi F, Shen X D, Gao F, Anselmo D, Ma J, Dry S, McDiarmid S V, Shaw G, Busuttil R W and Kupiec-Weglinski J (2002) Improved Survival Through the Reduction of Ischemia-Reperfusion Injury After Rat Intestinal Transplantation Using Selective P-Selectin Blockade With P-Selectin Glycoprotein Ligand-Ig. *Transplant Proc* **34**: pp 985.

Feld AD, Fondacaro J D, Holloway G A, Jr. and Jacobson E D (1982) Measurement of Mucosal Blood Flow in the Canine Intestine With Laser Doppler Velocimetry. *Life Sci* **31**: pp 1509-1517.

Ferencz A, Szanto Z, Borsiczky B, Kiss K, Kalmar-Nagy K, Szeberenyi J, Horvath P O and Roth E (2002) The Effects of Preconditioning on the Oxidative Stress in Small-Bowel Autotransplantation. *Surgery* **132**: pp 877-884.

Ferencz A, Szanto Z, Kalmar-Nagy K, Horvath O P and Roth E (2004) Mitigation of Oxidative Injury by Classic and Delayed Ischemic Preconditioning Prior to Small Bowel Autotransplantation. *Transplantation Proceedings* **36**: pp 286-288.

Ferrer JV, Ariceta J, Guerrero D, Gomis T, Larrea M M, Balen E and Lera J M (1998) Allopurinol and N-Acetylcysteine Avoid 60% of Intestinal Necrosis in an Ischemia-Reperfusion Experimental Model. *Transplant Proc* **30**: pp 2672.

Fleming SD, Starnes B W, Kiang J G, Stojadinovic A, Tsokos G C and Shea-Donohue T (2002) Heat Stress Protection Against Mesenteric I/R-Induced Alterations in Intestinal Mucosa in Rats. *J Appl Physiol* **92**: pp 2600-2607.

Floyd TF, Boroughs A, Garvey C, Dasher J, Ikeda C B, Sloviter H A and Ziegler M M (1987) Intestinal Ischemia: Treatment by Peritoneal Lavage With Oxygenated Perfluorochemical. *J Pediatr Surg* **22**: pp 1191-1197.

Foulds S, Cheshire N J, Schachter M, Wolfe J H and Mansfield A O (1997) Endotoxin Related Early Neutrophil Activation Is Associated With Outcome After Thoracoabdominal Aortic Aneurysm Repair. *Br J Surg* **84**: pp 172-177.

Fox-Robichaud A, Payne D, Hasan S U, Ostrovsky L, Fairhead T, Reinhardt P and Kubes P (1998) Inhaled NO As a Viable Antiadhesive Therapy for Ischemia/Reperfusion Injury of Distal Microvascular Beds. *J Clin Invest* **101**: pp 2497-2505.

Fujino Y, Suzuki Y, Kakinoki K, Tanioka Y, Ku Y and Kuroda Y (2003b) Protection Against Experimental Small Intestinal Ischaemia-Reperfusion Injury With Oxygenated Perfluorochemical. *Br J Surg* **90**: pp 1015-1020.

Fujino Y, Suzuki Y, Kakinoki K, Tanioka Y, Ku Y and Kuroda Y (2003a) Protection Against Experimental Small Intestinal Ischaemia-Reperfusion Injury With Oxygenated Perfluorochemical. *Br J Surg* **90**: pp 1015-1020.

Fukatsu K, Lundberg A H, Hanna M K, Wu Y, Wilcox H G, Granger D N, Gaber A O and Kudsk K A (1999) Route of Nutrition Influences Intercellular Adhesion Molecule-1 Expression and Neutrophil Accumulation in Intestine. *Arch Surg* **134**: pp 1055-1060.

Fukatsu K, Lundberg A H, Hanna M K, Wu Y, Wilcox H G, Granger D N, Gaber A O and Kudsk K A (2000) Increased Expression of Intestinal P-Selectin and Pulmonary E-Selectin During Intravenous Total Parenteral Nutrition. *Arch Surg* **135**: pp 1177-1182.

Fukatsu K, Zarzaur B L, Johnson C D, Lundberg A H, Wilcox H G and Kudsk K A (2001) Enteral Nutrition Prevents Remote Organ Injury and Death After a Gut Ischemic Insult. *Ann Surg* **233**: pp 660-668.

Gauthier TW, Davenpeck K L and Lefer A M (1994) Nitric Oxide Attenuates Leukocyte-Endothelial Interaction Via P-Selectin in Splanchnic Ischemia-Reperfusion. *Am J Physiol* **267**: pp G562-G568.

Geboes K, Geboes K P and Maleux G (2001) Vascular Anatomy of the Gastrointestinal Tract. *Best Pract Res Clin Gastroenterol* **15**: pp 1-14.

Ghosh S, Roberts N, Firmin R K, Jameson J and Spyt T J (2002) Risk Factors for Intestinal Ischaemia in Cardiac Surgical Patients. *Eur J Cardiothorac Surg* **21**: pp 411-416.

Gibbs SA, Weiser M R, Kobzik L, Valeri C R, Shepro D and Hechtman H B (1996) P-Selectin Mediates Intestinal Ischemic Injury by Enhancing Complement Deposition. *Surgery* **119**: pp 652-656.

Gidday JM, Park T S, Shah A R and Gonzales E R (1998) Modulation of Basal and Postischemic Leukocyte-Endothelial Adherence by Nitric Oxide. *Stroke* **29**: pp 1423-1429.

Glazier SS, O'Rourke D M, Graham D I and Welsh F A (1994) Induction of Ischemic Tolerance Following Brief Focal Ischemia in Rat Brain. *J Cereb Blood Flow Metab* **14**: pp 545-553.

Gonzalez AP, Sepulveda S, Massberg S, Baumeister R and Menger M D (1994) In Vivo Fluorescence Microscopy for the Assessment of Microvascular Reperfusion Injury in Small Bowel Transplants in Rats. *Transplantation* **58**: pp 403-408.

Granger DN (1988) Role of Xanthine Oxidase and Granulocytes in Ischemia-Reperfusion Injury. *Am J Physiol* **255**: pp H1269-H1275.

Granger DN, Hollwarth M E and Parks D A (1986) Ischemia-Reperfusion Injury: Role of Oxygen-Derived Free Radicals. *Acta Physiol Scand Suppl* **548**: pp 47-63.

Granger DN and Korthuis R J (1995) Physiologic Mechanisms of Postischemic Tissue Injury. *Annu Rev Physiol* **57**: pp 311-332.

Granger DN, Richardson P D, Kvietys P R and Mortillaro N A (1980) Intestinal Blood Flow. *Gastroenterology* **78**: pp 837-863.

Grisham MB, Granger D N and Lefer D J (1998) Modulation of Leukocyte-Endothelial Interactions by Reactive Metabolites of Oxygen and Nitrogen: Relevance to Ischemic Heart Disease. *Free Radic Biol Med* **25**: pp 404-433.

Grotz MR, Pape H C, van Griensven M, Stalp M, Rohde F, Bock D and Krettek C (2001) Glycine Reduces the Inflammatory Response and Organ Damage in a Two-Hit Sepsis Model in Rats. *Shock* **16**: pp 116-121.

Guimaraes FA, Taha M O, Simoes M J and Fagundes D J (2002) Ischemia-Reperfusion of the Small Intestine and Hyperbaric Oxygen Treatment: a Morphologic Study in Rats. *Transplant Proc* **34**: pp 977-979.

Gunel E, Caglayan F, Caglayan O, Dilsiz A, Duman S and Aktan M (1998) Treatment of Intestinal Reperfusion Injury Using Antioxidative Agents. *J Pediatr Surg* **33**: pp 1536-1539.

Guo JP, Murohara T, Buerke M, Scalia R and Lefer A M (1996) Direct Measurement of Nitric Oxide Release From Vascular Endothelial Cells. *J Appl Physiol* **81**: pp 774-779.

Halliwell B and Gutteridge J (1999) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford.

Hammerman C, Goldschmidt D, Caplan M S, Kaplan M, Bromiker R, Eidelman A I, Gartner L M and Hochman A (2002) Protective Effect of Bilirubin in Ischemia-Reperfusion Injury in the Rat Intestine. *J Pediatr Gastroenterol Nutr* **35**: pp 344-349.

Hammerman C, Goldschmidt D, Caplan M S, Kaplan M, Schimmel M S, Eidelman A I, Branski D and Hochman A (1999) Amelioration of Ischemia-Reperfusion Injury in Rat Intestine by Pentoxifylline-Mediated Inhibition of Xanthine Oxidase. *J Pediatr Gastroenterol Nutr* **29**: pp 69-74.

Hansell P and Ulfendahl H R (1986) Atriopeptins and Renal Cortical and Papillary Blood Flow. *Acta Physiol Scand* **127**: pp 349-357.

Harris NR and Granger D N (1997) Organ Procurements and Preservation for transplantation, in *Ischemia /Reperfusion Injury* pp 67-81, Springer-Verlag.

Harrison R (2002) Structure and Function of Xanthine Oxidoreductase: Where Are We Now? *Free Radic Biol Med* **33**: pp 774-797.

Hartsfield CL, Alam J and Choi A M (1998) Transcriptional Regulation of the Heme Oxygenase 1 Gene by Pyrrolidine Dithiocarbamate. *FASEB J* **12**: pp 1675-1682.

Harward TR, Coe D, Souba W W, Klingman N and Seeger J M (1994) Glutamine Preserves Gut Glutathione Levels During Intestinal Ischemia/Reperfusion. *J Surg Res* **56**: pp 351-355.

Hassoun HT, Zou L, Moore F A, Kozar R A, Weisbrodt N W and Kone B C (2002) Alpha-Melanocyte-Stimulating Hormone Protects Against Mesenteric Ischemia-Reperfusion Injury. *Am J Physiol Gastrointest Liver Physiol* **282**: pp G1059-G1068.

Hayashi S, Takamiya R, Yamaguchi T, Matsumoto K, Tojo S J, Tamatani T, Kitajima M, Makino N, Ishimura Y and Suematsu M (1999) Induction of Heme Oxygenase-1 Suppresses Venular Leukocyte Adhesion Elicited by Oxidative Stress: Role of Bilirubin Generated by the Enzyme. *Circ Res* **85**: pp 663-671.

Hayward R and Lefer A M (1998) Time Course of Endothelial-Neutrophil Interaction in Splanchnic Artery Ischemia-Reperfusion. *Am J Physiol* **275**: pp H2080-H2086.

Heller T, Hennecke M, Baumann U, Gessner J E, zu Vilsendorf A M, Baensch M, Boulay F, Kola A, Klos A, Bautsch W and Kohl J (1999) Selection of a C5a Receptor Antagonist From Phage Libraries Attenuating the Inflammatory Response in Immune Complex Disease and Ischemia/Reperfusion Injury. *J Immunol* **163**: pp 985-994.

Hernandez LA, Grisham M B and Granger D N (1987) A Role for Iron in Oxidant-Mediated Ischemic Injury to Intestinal Microvasculature. *Am J Physiol* **253**: pp G49-G53.

Heuser M, Pfaar O, Gralla O, Grone H J, Nustede R and Post S (2000) Impact of Gastrin-Releasing Peptide on Intestinal Microcirculation After Ischemia-Reperfusion in Rats. *Digestion* **61**: pp 172-180.

Hierholzer C, Kalff J C, Audolfsson G, Billiar T R, Tweardy D J and Bauer A J (1999) Molecular and Functional Contractile Sequelae of Rat Intestinal Ischemia/Reperfusion Injury. *Transplantation* **68**: pp 1244-1254.

Hinnebusch BF, Ma Q, Henderson J W, Siddique A, Archer S Y and Hodin R A (2002) Enterocyte Response to Ischemia Is Dependent on Differentiation State. *J Gastrointest Surg* **6**: pp 403-409.

Hirschi KK and D'Amore P A (1996) Pericytes in the Microvasculature. *Cardiovasc Res* **32**: pp 687-698.

Holloway GA, Jr. and Watkins D W (1977) Laser Doppler Measurement of Cutaneous Blood Flow. *J Invest Dermatol* **69**: pp 306-309.

Hosotani R, Inoue K and Tobe T (1985) [Measurement of Canine Pancreatic Blood Flow by Laser-Doppler Flowmetry: Blood Flow Response to Gut Hormones (VIP, PHI, GRP)]. *Nippon Shokakibyo Gakkai Zasshi* **82**: pp 1563-1571.

Hotchkiss R, Nunnally I, Lindquist S, Taulien J, Perdrizet G and Karl I (1993) Hyperthermia Protects Mice Against the Lethal Effects of Endotoxin. *Am J Physiol* **265**: pp R1447-R1457.

Hotter G, Closa D, Prados M, Fernandez-Cruz L, Prats N, Gelpi E and Rosello-Catafau J (1996) Intestinal Preconditioning Is Mediated by a Transient Increase in Nitric Oxide. *Biochem Biophys Res Commun* **222**: pp 27-32.

Iijima S, Shou J, Naama H, Calvano S E and Daly J M (1997) Beneficial Effect of Enteral Glycine in Intestinal Ischemia/Reperfusion Injury. *J Gastrointest Surg* **1**: pp 53-60.

Ikeda S, Zarzaur B L, Johnson C D, Fukatsu K and Kudsk K A (2002) Total Parenteral Nutrition Supplementation With Glutamine Improves Survival After Gut Ischemia/Reperfusion. *JPEN J Parenter Enteral Nutr* **26**: pp 169-173.

Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K and Masaki T (1989) The Human Endothelin Family: Three Structurally and Pharmacologically Distinct Isopeptides Predicted by Three Separate Genes. *Proc Natl Acad Sci U S A* **86**: pp 2863-2867.

Ito K, Ozasa H, Kojima N, Miura M, Iwa T, Senoo H and Horikawa S (2003) Pharmacological Preconditioning Protects Lung Injury Induced by Intestinal Ischemia/Reperfusion in Rat. *Shock* **19**: pp 462-468.

Jacob T, Ascher E, Hingorani A and Kallakuri S (2003) Glycine Prevents the Induction of Apoptosis Attributed to Mesenteric Ischemia/Reperfusion Injury in a Rat Model. *Surgery* **134**: pp 457-466.

Jassem W, Fuggle S V, Rela M, Koo D D and Heaton N D (2002) The Role of Mitochondria in Ischemia/Reperfusion Injury. *Transplantation* **73**: pp 493-499.

Jefayri MK, Grace P A and Mathie R T (2000) Attenuation of Reperfusion Injury by Renal Ischaemic Preconditioning: the Role of Nitric Oxide. *BJU Int* **85**: pp 1007-1013.

Jenkins DP, Pugsley W B, Alkhulaifi A M, Kemp M, Hooper J and Yellon D M (1997) Ischaemic Preconditioning Reduces Troponin T Release in Patients Undergoing Coronary Artery Bypass Surgery. *Heart* **77**: pp 314-318.

Jobsis FF (1977) Noninvasive, Infrared Monitoring of Cerebral and Myocardial Oxygen Sufficiency and Circulatory Parameters. *Science* **198**: pp 1264-1267.

Johnson PC (1960) Autoregulation of Intestinal Blood Flow. *Am J Physiol* **199**: pp 311-318.

Kalia N, Brown N J, Hopkinson K, Stephenson T J, Wood R F and Pockley A G (2002) FK409 Inhibits Both Local and Remote Organ Damage After Intestinal Ischaemia. *J Pathol* **197**: pp 595-602.

Kaminski PM and Proctor K G (1989) Attenuation of No-Reflow Phenomenon, Neutrophil Activation, and Reperfusion Injury in Intestinal Microcirculation by Topical Adenosine. *Circ Res* **65**: pp 426-435.

Karpel-Massler G, Fleming S D, Kirschfink M and Tsokos G C (2003) Human C1 Esterase Inhibitor Attenuates Murine Mesenteric Ischemia/Reperfusion Induced Local Organ Injury. *J Surg Res* **115**: pp 247-256.

Kasten FH (1993) *Introduction to Fluorescent Probes: Properties, History and Applications.* Academic Press Limited, London.

Katori M, Anselmo D M, Busuttil R W and Kupiec-Weglinski J W (2002a) A Novel Strategy Against Ischemia and Reperfusion Injury: Cytoprotection With Heme Oxygenase System. *Transpl Immunol* **9**: pp 227-233.

Katori M, Busuttil R W and Kupiec-Weglinski J W (2002b) Heme Oxygenase-1 System in Organ Transplantation. *Transplantation* **74**: pp 905-912.

Kawata K, Takeyoshi I, Iwanami K, Sunose Y, Aiba M, Ohwada S, Matsumoto K and Morishita Y (2001) A Spontaneous Nitric Oxide Donor Ameliorates Small Bowel Ischemia-Reperfusion Injury in Dogs. *Dig Dis Sci* **46**: pp 1748-1756.

Kazez A, Demirbag M, Ustundag B, Ozercan I H and Saglam M (2000) The Role of Melatonin in Prevention of Intestinal Ischemia-Reperfusion Injury in Rats. *J Pediatr Surg* **35**: pp 1444-1448.

Kelly D, Piasecki C, Anthony A, Dhillon A P, Pounder R E and Wakefield A J (1998) Reversal and Protection Against Indomethacin-Induced Blood Stasis and Mucosal Damage in the Rat Jejunum by a Beta3-Adrenoceptor Agonist. *Aliment Pharmacol Ther* **12**: pp 1121-1129.

Kelly D and Wischmeyer P E (2003) Role of L-Glutamine in Critical Illness: New Insights. *Curr Opin Clin Nutr Metab Care* **6**: pp 217-222.

Kelly D, Zhong Z, Wheeler M D, Li X, Froh M, Schemmer P, Yin M, Bunzendaul H, Bradford B and Lemasters J J (2003) L-Glycine: a Novel Antiinflammatory, Immunomodulatory, and Cytoprotective Agent. *Curr Opin Clin Nutr Metab Care* **6**: pp 229-240.

Khanna A, Rossman J, Caty M G and Fung H L (2003) Beneficial Effects of Intraluminal Nitroglycerin in Intestinal Ischemia-Reperfusion Injury in Rats. *J Surg Res* **114**: pp 15-24.

Kimizuka K, Nakao A, Nalesnik M A, Demetris A J, Uchiyama T, Ruppert K, Fink M P, Stolz D B and Murase N (2004) Exogenous IL-6 Inhibits Acute Inflammatory Responses and Prevents Ischemia/Reperfusion Injury After Intestinal Transplantation. *Am J Transplant* **4**: pp 482-494.

Kimura T, Andoh A, Fujiyama Y, Saotome T and Bamba T (1998) A Blockade of Complement Activation Prevents Rapid Intestinal Ischaemia-Reperfusion Injury by Modulating Mucosal Mast Cell Degranulation in Rats. *Clin Exp Immunol* **111**: pp 484-490.

Koike K, Moore F A, Moore E E, Read R A, Carl V S and Banerjee A (1993) Gut Ischemia Mediates Lung Injury by a Xanthine Oxidase-Dependent Neutrophil Mechanism. *J Surg Res* **54**: pp 469-473.

Kojima M, Iwakiri R, Wu B, Fujise T, Watanabe K, Lin T, Amemori S, Sakata H, Shimoda R, Oguzu T, Ootani A, Tsunada S and Fujimoto K (2003) Effects of Antioxidative Agents on Apoptosis Induced by Ischaemia-Reperfusion in Rat Intestinal Mucosa. *Aliment Pharmacol Ther* **18 Suppl 1:139-45.**: pp 139-145.

Koksoy C, Kuzu M A, Kuzu I, Ergun H and Gurhan I (2001) Role of Tumour Necrosis Factor in Lung Injury Caused by Intestinal Ischaemia-Reperfusion. *Br J Surg* **88**: pp 464-468.

Kong SE, Blennerhassett L R, Heel K A, McCauley R D and Hall J C (1998) Ischaemia-Reperfusion Injury to the Intestine. *Aust N Z J Surg* **68**: pp 554-561.

Kosonen O, Kankaanranta H, Malo-Ranta U and Moilanen E (1999) Nitric Oxide-Releasing Compounds Inhibit Neutrophil Adhesion to Endothelial Cells. *Eur J Pharmacol* **382**: pp 111-117.

Koti RS, Seifalian A M, McBride A G, Yang W and Davidson B R (2002a) The Relationship of Hepatic Tissue Oxygenation With Nitric Oxide Metabolism in Ischemic Preconditioning of the Liver. *FASEB J* **16**: pp 1654-1656.

Koti RS, Yang W, Dashwood M R, Davidson B R and Seifalian A M (2002b) Effect of Ischemic Preconditioning on Hepatic Microcirculation and Function in a Rat Model of Ischemia Reperfusion Injury. *Liver Transpl* **8**: pp 1182-1191.

Koyama T, Mishina H and Asakura T (1975) Study of Micro-Circulation in Web of Frog (Xenous Laevis Daudin) by Using Laser Doppler Microscope. *Experientia* **31**: pp 1420-1422.

Kubes P and McCafferty D M (2000) Nitric Oxide and Intestinal Inflammation. *Am J Med* **109**: pp 150-158.

Kubes P, Payne D and Ostrovsky L (1998) Preconditioning and Adenosine in I/R-Induced Leukocyte-Endothelial Cell Interactions. *Am J Physiol* **274**: pp H1230-H1238.

Kuenzler KA, Pearson P Y and Schwartz M Z (2002a) Hepatoctye Growth Factor Pretreatment Reduces Apoptosis and Mucosal Damage After Intestinal Ischemia-Reperfusion. *J Pediatr Surg* **37**: pp 1093-discussion.

Kuenzler KA, Pearson P Y and Schwartz M Z (2002b) Interleukin-11 Enhances Intestinal Absorptive Function After Ischemia-Reperfusion Injury. *J Pediatr Surg* **37**: pp 457-459.

Lacey JM and Wilmore D W (1990) Is Glutamine a Conditionally Essential Amino Acid? *Nutrition Reviews* **48**: pp 297-309.

Lanciault G and Jacobson E D (1976) The Gastrointestinal Circulation. *Gastroenterology* **71**: pp 851-873.

Lane JS, Todd K E, Lewis M P, Gloor B, Ashley S W, Reber H A, McFadden D W and Chandler C F (1997) Interleukin-10 Reduces the Systemic Inflammatory Response in a Murine Model of Intestinal Ischemia/Reperfusion. *Surgery* **122**: pp 288-294.

Lee MA, McCauley R D, Kong S E and Hall J C (2001) Pretreatment With Glycine Reduces the Severity of Warm Intestinal Ischemic-Reperfusion Injury in the Rat. *Ann Plast Surg* **46**: pp 320-326.

Lee MA, McCauley R D, Kong S E and Hall J C (2002) Influence of Glycine on Intestinal Ischemia-Reperfusion Injury. *JPEN J Parenter Enteral Nutr* **26**: pp 130-135.

Lee PJ, Jiang B H, Chin B Y, Iyer N V, Alam J, Semenza G L and Choi A M (1997) Hypoxia-Inducible Factor-1 Mediates Transcriptional Activation of the Heme Oxygenase-1 Gene in Response to Hypoxia. *J Biol Chem* **272**: pp 5375-5381.

Lefer AM and Lefer D J (1996) The Role of Nitric Oxide and Cell Adhesion Molecules on the Microcirculation in Ischaemia-Reperfusion. *Cardiovasc Res* **32**: pp 743-751.

Lelli JL, Jr., Pradhan S and Cobb L M (1993) Prevention of Postischemic Injury in Immature Intestine by Deferoxamine. *J Surg Res* **54**: pp 34-38.

Li Y, Roth S, Laser M, Ma J X and Crosson C E (2003) Retinal Preconditioning and the Induction of Heat-Shock Protein 27. *Invest Ophthalmol Vis Sci* **44**: pp 1299-1304.

Li YS, Li J S, Jiang J W, Liu F N, Li N, Qin W S and Zhu H (1999) Glycyl-Glutamine-Enriched Long-Term Total Parenteral Nutrition Attenuates Bacterial Translocation Following Small Bowel Transplantation in the Pig. *J Surg Res* **82**: pp 106-111.

Liu SF, Ye X and Malik A B (1999) Inhibition of NF-KappaB Activation by Pyrrolidine Dithiocarbamate Prevents In Vivo Expression of Proinflammatory Genes. *Circulation* **100**: pp 1330-1337.

Loong CC, Chiu J H, Tiao R C, Chiu Y Y, Wu C W and Lui W Y (2001) Pretreatment With Magnolol Attenuates Ischemia-Reperfusion Injury in Rat Small Intestine. *Transplant Proc* **33**: pp 3737-3738.

Luo CC, Chen H M, Chiu C H, Lin J N and Chen J C (2001) Effect of N(G)-Nitro-L-Arginine Methyl Ester on Intestinal Permeability Following Intestinal Ischemia-Reperfusion Injury in a Rat Model. *Biol Neonate* **80**: pp 60-63.

Maines MD (1988) Heme Oxygenase: Function, Multiplicity, Regulatory Mechanisms, and Clinical Applications. *FASEB J* **2**: pp 2557-2568.

Mallick IH, Yang W, Winslet M C and Seifalian A M (2004) Ischemia-Reperfusion Injury of the Intestine and Protective Strategies Against Injury. *Dig Dis Sci* **49**: pp 1359-1377.

Marshall JC (1998) The Gut As a Potential Trigger of Exercise-Induced Inflammatory Responses. *Can J Physiol Pharmacol* **76**: pp 479-484.

Massberg S, Gonzalez A P, Leiderer R, Menger M D and Messmer K (1998) In Vivo Assessment of the Influence of Cold Preservation Time on Microvascular Reperfusion Injury After Experimental Small Bowel Transplantation. *Br J Surg* **85**: pp 127-133.

Mazzon E, Dugo L, De S A, Li J H, Caputi A P, Zhang J and Cuzzocrea S (2002) Beneficial Effects of GPI 6150, an Inhibitor of Poly(ADP-Ribose) Polymerase in a Rat Model of Splanchnic Artery Occlusion and Reperfusion. *Shock* **17**: pp 222-227.

Mbachu EM, Klein L V, Rubin B B and Lindsay T F (2004) A Monoclonal Antibody Against Cytokine-Induced Neutrophil Chemoattractant Attenuates Injury in the Small Intestine in a Model of Ruptured Abdominal Aortic Aneurysm. *J Vasc Surg* **39**: pp 1104-1111.

McCallion K, Wattanasirichaigoon S, Gardiner K R and Fink M P (2000) Ischemic Preconditioning Ameliorates Ischemia- and Reperfusion-Induced Intestinal Epithelial Hyperpermeability in Rats. *Shock* **14**: pp 429-434.

Meneshian A and Bulkley G B (2002) The Physiology of Endothelial Xanthine Oxidase: From Urate Catabolism to Reperfusion Injury to Inflammatory Signal Transduction. *Microcirculation* **9**: pp 161-175.

Mester MD, Tompkins M D, Gelfand M D and Dinarello M D (1993) Intestinal Production of Interleukin-1[Alpha] During Endotoxemia in the Mouse. *J Surg Res* **54**: pp 584-591.

Meyer TA, Wang J, Tiao G M, Ogle C K, Fischer J E and Hasselgren P O (1995) Sepsis and Endotoxemia Stimulate Intestinal Interleukin-6 Production. *Surgery* **118**: pp 336-342.

Mocan H, Gedik Y, Erduran E, Mocan Z M, Okten A, Gacar N and Pul N (1995) The Role of Calcium Channel Entry Blocker in Experimental Ischemia-Reperfusion-Induced Intestinal Injury. *Pol J Pharmacol* **47**: pp 179-183.

Molmenti EP, Ziambaras T and Perlmutter D H (1993) Evidence for an Acute Phase Response in Human Intestinal Epithelial Cells. *J Biol Chem* **268**: pp 14116-14124.

Montalto MC, Hart M L, Jordan J E, Wada K and Stahl G L (2003) Role for Complement in Mediating Intestinal Nitric-Oxide Synthase-2 and Superoxide Dismutase Expression. *Am J Physiol Gastrointest Liver Physiol.*

Moore EE, Moore F A, Franciose R J, Kim F J, Biffl W L and Banerjee A (1994) The Postischemic Gut Serves As a Priming Bed for Circulating Neutrophils That Provoke Multiple Organ Failure. *J Trauma* **37**: pp 881-887.

Motterlini R, Foresti R, Intaglietta M and Winslow R M (1996) NO-Mediated Activation of Heme Oxygenase: Endogenous Cytoprotection Against Oxidative Stress to Endothelium. *Am J Physiol* **270**: pp H107-H114.

Murry CE, Jennings R B and Reimer K A (1986) Preconditioning With Ischemia: a Delay of Lethal Cell Injury in Ischemic Myocardium. *Circulation* **74**: pp 1124-1136.

Naito Y, Takagi T, Ichikawa H, Tomatsuri N, Kuroda M, Isozaki Y, Katada K, Uchiyama K, Kokura S, Yoshida N, Okanoue T and Yoshikawa T (2004) A Novel Potent Inhibitor of Inducible Nitric Oxide Inhibitor, ONO-1714, Reduces Intestinal Ischemia-Reperfusion Injury in Rats. *Nitric Oxide* **10**: pp 170-177.

Naito Y, Takagi T, Uchiyama K, Handa O, Tomatsuri N, Imamoto E, Kokura S, Ichikawa H, Yoshida N and Yoshikawa T (2002) Suppression of Intestinal Ischemia-Reperfusion Injury by a Specific Peroxisome Proliferator-Activated Receptor-Gamma Ligand, Pioglitazone, in Rats. *Redox Rep* **7**: pp 294-299.

Nakamura M, Ozaki M, Fuchinoue S, Teraoka S and Ota K (1997) Ascorbic Acid Prevents Ischemia-Reperfusion Injury in the Rat Small Intestine. *Transpl Int* **10**: pp 89-95.

Nakamura N, Hamada N, Murata R, Kobayashi A, Ishizaki N, Taira A and Sakata R (2001) Contribution of Serotonin to Liver Injury Following Canine Small-Intestinal Ischemia and Reperfusion. *J Surg Res* **99**: pp 17-24.

Nakao A, Kimizuka K, Stolz D B, Neto J S, Kaizu T, Choi A M, Uchiyama T, Zuckerbraun B S, Nalesnik M A, Otterbein L E and Murase N (2003) Carbon Monoxide Inhalation Protects Rat Intestinal Grafts From Ischemia/Reperfusion Injury. *Am J Pathol* **163**: pp 1587-1598.

Nalini S, Mathan M M and Balasubramanian K A (1993) Oxygen Free Radical Induced Damage During Intestinal Ischemia/Reperfusion in Normal and Xanthine Oxidase Deficient Rats. *Mol Cell Biochem* **124**: pp 59-66.

Nikon. Epifluorescence Microscope manual. 2004. Nikon.
Ref Type: Pamphlet

O'Donnell KA, Caty M G, Zheng S, Rossman J E and Azizkhan R G (1997) Oxygenated Intraluminal Perfluorocarbon Protects Intestinal Muscosa From Ischemia/Reperfusion Injury. *J Pediatr Surg* **32**: pp 361-365.

Ogle CK, Mao J X, Wu J Z, Ogle J D and Alexander J W (1994) The 1994 Lindberg Award. The Production of Tumor Necrosis Factor, Interleukin-1, Interleukin-6, and Prostaglandin E2 by Isolated Enterocytes and Gut Macrophages: Effect of Lipopolysaccharide and Thermal Injury. *J Burn Care Rehabil* **15**: pp 470-477.

Ohara M, Unno N, Mitsuoka H, Kaneko H and Nakamura S (2001) Peritoneal Lavage With Oxygenated Perfluorochemical Preserves Intestinal Mucosal Barrier Function After Ischemia-Reperfusion and Ameliorates Lung Injury. *Crit Care Med* **29**: pp 782-788.

Ohlmann A, Giffhorn-Katz S, Becker I, Katz N and Immenschuh S (2003) Regulation of Heme Oxygenase-1 Gene Expression by Anoxia and Reoxygenation in Primary Rat Hepatocyte Cultures. *Exp Biol Med (Maywood)* **228**: pp 584-589.

Oktar BK, Coskun T, Bozkurt A, Yegen B C, Yuksel M, Haklar G, Bilsel S, Aksungar F B, Cetinel U, Granger D N and Kurtel H (2000) Endothelin-1-Induced PMN Infiltration and Mucosal Dysfunction in the Rat Small Intestine. *Am J Physiol Gastrointest Liver Physiol* **279**: pp G483-G491.

Oktar BK, Gulpinar M A, Bozkurt A, Ghandour S, Cetinel S, Moini H, Yegen B C, Bilsel S, Granger D N and Kurtel H (2002) Endothelin Receptor Blockers Reduce I/R-Induced Intestinal Mucosal Injury: Role of Blood Flow. *Am J Physiol Gastrointest Liver Physiol* **282**: pp G647-G655.

Oldham KT, Guice K S, Gore D, Gourley W K and Lobe T E (1987) Treatment of Intestinal Ischemia With Oxygenated Intraluminal Perfluorocarbons. *Am J Surg* **153**: pp 291-294.

Ozden A, Tetik C, Bilgihan A, Calli N, Bostanci B, Yis O and Duzcan E (1999) Antithrombin III Prevents 60 Min Warm Intestinal Ischemia Reperfusion Injury in Rats. *Res Exp Med (Berl)* **198**: pp 237-246.

Ozel SK, Yuksel M, Haklar G, Durakbasa C U, Dagli T E and Aktan A O (2001) Nitric Oxide and Endothelin Relationship in Intestinal Ischemia/Reperfusion Injury (II). *Prostaglandins Leukot Essent Fatty Acids* **64**: pp 253-257.

Panes J, Perry M and Granger D N (1999) Leukocyte-Endothelial Cell Adhesion: Avenues for Therapeutic Intervention. *Br J Pharmacol* **126**: pp 537-550.

Pang CY, Neligan P, Zhong A, He W, Xu H and Forrest C R (1997) Effector Mechanism of Adenosine in Acute Ischemic Preconditioning of Skeletal Muscle Against Infarction. *Am J Physiol* **273**: pp R887-R895.

Papanastasiou S, Estdale S E, Homer-Vanniasinkam S and Mathie R T (1999) Protective Effect of Preconditioning and Adenosine Pretreatment in Experimental Skeletal Muscle Reperfusion Injury. *Br J Surg* **86**: pp 916-922.

Parks DA, Williams T K and Beckman J S (1988) Conversion of Xanthine Dehydrogenase to Oxidase in Ischemic Rat Intestine: a Reevaluation. *Am J Physiol* **254**: pp G768-G774.

Pastores SM, Katz D P and Kvetan V (1996) Splanchnic Ischemia and Gut Mucosal Injury in Sepsis and the Multiple Organ Dysfunction Syndrome. *Am J Gastroenterol* **91**: pp 1697-1710.

Patel A, van de Poll M C, Greve J W, Buurman W A, Fearon K C, McNally S J, Harrison E M, Ross J A, Garden O J, Dejong C H and Wigmore S J (2004) Early Stress Protein Gene Expression in a Human Model of Ischemic Preconditioning. *Transplantation* **78**: pp 1479-1487.

Peng HB, Libby P and Liao J K (1995) Induction and Stabilization of I Kappa B Alpha by Nitric Oxide Mediates Inhibition of NF-Kappa B. *J Biol Chem* **270**: pp 14214-14219.

Prasad R, Alavi K and Schwartz M Z (2001) GLP-2alpha Accelerates Recovery of Mucosal Absorptive Function After Intestinal Ischemia/Reperfusion. *J Pediatr Surg* **36**: pp 570-572.

Puglisi RN, Strande L, Santos M, Doolin E J, Hewitt C W and Whalen T V (1996) The Effect of Cyclosporine in Gut Ischemic Injury: a Computerized Morphometric and Enzymatic Analysis. *J Pediatr Surg* **31**: pp 319-322.

Puisieux F, Deplanque D, Pu Q, Souil E, Bastide M and Bordet R (2000) Differential Role of Nitric Oxide Pathway and Heat Shock Protein in Preconditioning and Lipopolysaccharide-Induced Brain Ischemic Tolerance. *Eur J Pharmacol* **389**: pp 71-78.

Qu XW, Wang H, De Plaen I G, Rozenfeld R A and Hsueh W (2001) Neuronal Nitric Oxide Synthase (NOS) Regulates the Expression of Inducible NOS in Rat Small Intestine Via Modulation of Nuclear Factor Kappa B. *FASEB J* **15**: pp 439-446.

Rajeevprasad R, Alavi K and Schwartz M Z (2000) Glucagonlike Peptide-2 Analogue Enhances Intestinal Mucosal Mass and Absorptive Function After Ischemia-Reperfusion Injury. *J Pediatr Surg* **35**: pp 1537-1539.

Reber PU, Peter M, Patel A G, Stauffer E, Printzen G, Mettler D, Hakki H and Kniemeyer H W (2001) Ischaemia/Reperfusion Contributes to Colonic Injury Following Experimental Aortic Surgery. *Eur J Vasc Endovasc Surg* **21**: pp 35-39.

Riaz AA, Wan M X, Schaefer T, Schramm R, Ekberg H, Menger M D, Jeppsson B and Thorlacius H (2002a) Fundamental and Distinct Roles of P-Selectin and LFA-1 in

Ischemia/Reperfusion-Induced Leukocyte-Endothelium Interactions in the Mouse Colon. *Ann Surg* **236**: pp 777-784.

Riaz AA, Wan M X, Schafer T, Dawson P, Menger M D, Jeppsson B and Thorlacius H (2002b) Allopurinol and Superoxide Dismutase Protect Against Leucocyte-Endothelium Interactions in a Novel Model of Colonic Ischaemia-Reperfusion. *Br J Surg* **89**: pp 1572-1580.

Ricci JL, Sloviter H A and Ziegler M M (1985) Intestinal Ischemia: Reduction of Mortality Utilizing Intraluminal Perfluorochemical. *Am J Surg* **149**: pp 84-90.

Richard V, Kaeffer N, Tron C and Thuillez C (1994) Ischemic Preconditioning Protects Against Coronary Endothelial Dysfunction Induced by Ischemia and Reperfusion. *Circulation* **89**: pp 1254-1261.

Riva C, Ross B and Benedek G B (1972) Laser Doppler Measurements of Blood Flow in Capillary Tubes and Retinal Arteries. *Invest Ophthalmol* **11**: pp 936-944.

Ryan AJ, Flanagan S W, Moseley P L and Gisolfi C V (1992) Acute Heat Stress Protects Rats Against Endotoxin Shock. *J Appl Physiol* **73**: pp 1517-1522.

Sakawaki T, Sasaki K and Hirata K (1998) Saline With Glutamine Improves Cold Preserved Small Bowel Graft Mortality. *Transplant Proc* **30**: pp 3471-3474.

Sakurai M, Hayashi T, Abe K, Aoki M, Sadahiro M and Tabayashi K (1998) Enhancement of Heat Shock Protein Expression After Transient Ischemia in the Preconditioned Spinal Cord of Rabbits. *J Vasc Surg* **27**: pp 720-725.

Salzman AL (1995) Nitric Oxide in the Gut. *New Horiz* **3**: pp 352-364.

Scolapio JS and Fleming C R (1998) Short Bowel Syndrome. *Gastroenterol Clin North Am* **27**: pp 467-79, viii.

Sener G, Akgun U, Satiroglu H, Topaloglu U and Keyer-Uysal M (2001) The Effect of Pentoxifylline on Intestinal Ischemia/Reperfusion Injury. *Fundam Clin Pharmacol* **15**: pp 19-22.

Shashikant MP, Badellino M M, Cooper B, Shaffer T H, Myers S I and Wolfson M R (2002) Physicochemical Properties of Perfluorochemical Liquids Influence Ventilatory Requirements, Pulmonary Mechanics, and Microvascular Permeability During Partial Liquid Ventilation Following Intestinal Ischemia/Reperfusion Injury. *Crit Care Med* **30**: pp 2300-2305.

Shepherd AP and Riedel G L (1982) Continuous Measurement of Intestinal Mucosal Blood Flow by Laser-Doppler Velocimetry. *Am J Physiol* **242**: pp G668-G672.

Siebenlist U, Franzoso G and Brown K (1994) Structure, Regulation and Function of NF-Kappa B. *Annu Rev Cell Biol* **10**: pp 405-455.

Sileri P, Morini S, Schena S, Rastellini C, Abcarian H, Benedetti E and Cicalese L (2002) Intestinal Ischemia/Reperfusion Injury Produces Chronic Abnormalities of Absorptive Function. *Transplant Proc* **34**: pp 984.

Sileri P, Sica G, Gentileschi P, Venza M, Manzelli A, Palmieri G, Spagnoli L G, Testa G, Benedetti E and Gaspari A L (2004) Ischemic Preconditioning Protects Intestine From Prolonged Ischemia. *Transplant Proc* **36**: pp 283-285.

Simpson R, Alon R, Kobzik L, Valeri C R, Shepro D and Hechtman H B (1993) Neutrophil and Nonneutrophil-Mediated Injury in Intestinal Ischemia-Reperfusion. *Ann Surg* **218**: pp 444-453.

Sisley AC, Desai T, Harig J M and Gewertz B L (1994) Neutrophil Depletion Attenuates Human Intestinal Reperfusion Injury. *J Surg Res* **57**: pp 192-196.

Slavikova H, Lojek A, Hamar J, Dukova M, Kubala L, Vondraek J and i M (1998) Total Antioxidant Capacity of Serum Increased in Early but Not Late Period After Intestinal Ischemia in Rats. *Free Radic Biol Med* **25**: pp 9-18.

Sola A, De Oca J, Gonzalez R, Prats N, Rosello-Catafau J, Gelpi E, Jaurrieta E and Hotter G (2001) Protective Effect of Ischemic Preconditioning on Cold Preservation and Reperfusion Injury Associated With Rat Intestinal Transplantation. *Ann Surg* **234**: pp 98-106.

Sola A, Hotter G, Prats N, Xaus C, Gelpi E and Rosello-Catafau J (2000) Modification of Oxidative Stress in Response to Intestinal Preconditioning. *Transplantation* **69**: pp 767-772.

Souza DG, Cara D C, Cassali G D, Coutinho S F, Silveira M R, Andrade S P, Poole S P and Teixeira M M (2000a) Effects of the PAF Receptor Antagonist UK74505 on Local and Remote Reperfusion Injuries Following Ischaemia of the Superior Mesenteric Artery in the Rat. *Br J Pharmacol* **131**: pp 1800-1808.

Souza DG, Cassali G D, Poole S and Teixeira M M (2001) Effects of Inhibition of PDE4 and TNF-Alpha on Local and Remote Injuries Following Ischaemia and Reperfusion Injury. *Br J Pharmacol* **134**: pp 985-994.

Souza DG, Coutinho S F, Silveira M R, Cara D C and Teixeira M M (2000b) Effects of a BLT Receptor Antagonist on Local and Remote Reperfusion Injuries After Transient Ischemia of the Superior Mesenteric Artery in Rats. *Eur J Pharmacol* **403**: pp 121-128.

Souza DG, Mendonca V A, de A C M, Poole S and Teixeira M M (2002) Role of Tachykinin NK Receptors on the Local and Remote Injuries Following Ischaemia and Reperfusion of the Superior Mesenteric Artery in the Rat. *Br J Pharmacol* **135**: pp 303-312.

Spiecker M, Darius H, Kaboth K, Hubner F and Liao J K (1998) Differential Regulation of Endothelial Cell Adhesion Molecule Expression by Nitric Oxide Donors and Antioxidants. *J Leukoc Biol* **63**: pp 732-739.

Squiers EC, Bruch D, Buelow R and Tice D G (1999) Pretreatment of Small Bowel Isograft Donors With Cobalt-Protoporphyrin Decreases Preservation Injury. *Transplant Proc* **31**: pp 585-586.

Stallion A, Kou T D, Miller K A, Dahms B B, Dudgeon D L and Levine A D (2002) IL-10 Is Not Protective in Intestinal Ischemia Reperfusion Injury. *J Surg Res* **105**: pp 145-152.

Stocker R, Yamamoto Y, McDonagh A F, Glazer A N and Ames B N (1987) Bilirubin Is an Antioxidant of Possible Physiological Importance. *Science* **235**: pp 1043-1046.

Stojadinovic A, Kiang J, Smallridge R, Galloway R and Shea-Donohue T (1995) Induction of Heat-Shock Protein 72 Protects Against Ischemia/Reperfusion in Rat Small Intestine. *Gastroenterology* **109**: pp 505-515.

Stroh R, Christopher T A, Lopez B L, Guo Y P, Amico-Roxas M and Ma X L (1998) L-Propionyl Carnitine, an Endogenous Ester in Fatty Acid Metabolism, Exerts Anti-Shock and Endothelial Protective Effects in Rat Splanchnic Ischemia-Reperfusion Injury. *Shock* **9**: pp 216-222.

Suematsu M, Wakabayashi Y and Ishimura Y (1996) Gaseous Monoxides: a New Class of Microvascular Regulator in the Liver. *Cardiovasc Res* **32**: pp 679-686.

Sun Z, Olanders K, Lasson A, Dib M, Annborn M, Andersson K, Wang X and Andersson R (2002) Effective Treatment of Gut Barrier Dysfunction Using an Antioxidant, a PAF Inhibitor, and Monoclonal Antibodies Against the Adhesion Molecule PECAM-1. *J Surg Res* **105**: pp 220-233.

Suzuki Y, Deitch E A, Mishima S, Lu Q and Xu D (2000) Inducible Nitric Oxide Synthase Gene Knockout Mice Have Increased Resistance to Gut Injury and Bacterial Translocation After an Intestinal Ischemia-Reperfusion Injury. *Crit Care Med* **28**: pp 3692-3696.

Swank GM and Deitch E A (1996) Role of the Gut in Multiple Organ Failure: Bacterial Translocation and Permeability Changes. *World J Surg* **20**: pp 411-417.

Sykes MK, Vickers M D, Hull C J, Winternburn.P.J. and Shepstone.B.J. (1991) Electromagnetic Radiation and Optical Measurements, in *Principles of Measurement and*

Monitoring in Anaesthesia and Intensive Care pp 92-105, Blackwell Scientific Publications, Oxford, UK.

Takeyoshi I, Zhang S, Nakamura K, Ikoma A, Zhu Y, Starzl T E and Todo S (1996) Effect of Ischemia on the Canine Large Bowel: a Comparison With the Small Intestine. *J Surg Res* **62**: pp 41-48.

Tamaki T, Konoeda Y, Yasuhara M, Tanaka M, Yokota N, Hayashi T, Katori M, Uchida Y and Kawamura A (1999) Glutamine-Induced Heme Oxygenase-1 Protects Intestines and Hearts From Warm Ischemic Injury. *Transplant Proc* **31**: pp 1018-1019.

Tamion F, Richard V, Lacoume Y and Thuillez C (2002) Intestinal Preconditioning Prevents Systemic Inflammatory Response in Hemorrhagic Shock. Role of HO-1. *Am J Physiol Gastrointest Liver Physiol* **283**: pp G408-G414.

Tetik C, Ozden A, Calli N, Bilgihan A, Bostanci B, Yis O and Bayramoglu H (1999) Cytoprotective Effect of Trimetazidine on 60 Minutes of Intestinal Ischemia-Reperfusion Injury in Rats. *Transpl Int* **12**: pp 108-112.

Thollander M, Hellstrom P M and Gazelius B (1997) Semi-Invasive Laser-Doppler Flowmetry Technique. New Application for Recordings of Hemodynamics in Combination With Manometry of Human Small Intestine. *Int J Microcirc Clin Exp* **17**: pp 15-21.

Thollander M, Hellstrom P M, Svensson T H and Gazelius B (1996) Haemodynamic Changes in the Small Intestine Correlate to Migrating Motor Complex in Humans. *Eur J Gastroenterol Hepatol* **8**: pp 777-785.

Thompson JS, Bragg L E and West W W (1990) Serum Enzyme Levels During Intestinal Ischemia. *Ann Surg* **211**: pp 369-373.

Tomatsuri N, Yoshida N, Takagi T, Katada K, Isozaki Y, Imamoto E, Uchiyama K, Kokura S, Ichikawa H, Naito Y, Okanoue T and Yoshikawa T (2004) Edaravone, a Newly Developed

Radical Scavenger, Protects Against Ischemia-Reperfusion Injury of the Small Intestine in Rats. *Int J Mol Med* **13**: pp 105-109.

Tsuchihashi S, Tamaki T, Tanaka M, Kawamura A, Kaizu T, Ikeda A and Kakita A (2003) Pyrrolidine Dithiocarbamate Provides Protection Against Hypothermic Preservation and Transplantation Injury in the Rat Liver: the Role of Heme Oxygenase-1. *Surgery* **133**: pp 556-567.

Tsuruma T, Yagihashi A, Hirata K, Matsuno T, Zou X M, Sasaki K, Asanuma K and Endo T (1996a) Evaluation of Plasma IL-8 (CINC) Concentration During Ischemia and After Reperfusion in the Small Intestine. *Transplant Proc* **28**: pp 1917-1918.

Tsuruma T, Yagihashi A, Matsuno T, Zou X M, Asanuma K, Sasaki K and Hirata K (1996b) The Heat-Shock Protein 70 Family Reduces Ischemia/Reperfusion Injury in Small Intestine. *Transplant Proc* **28**: pp 2629-2630.

Tsuruma T, Yagihashi A, Watanabe N, Yajima T, Kameshima H, Araya J and Hirata K (1999) Heat-Shock Protein-73 Protects Against Small Intestinal Warm Ischemia-Reperfusion Injury in the Rat. *Surgery* **125**: pp 385-395.

Turman MA and Bates C M (1997) Susceptibility of Human Proximal Tubular Cells to Hypoxia: Effect of Hypoxic Preconditioning and Comparison to Glomerular Cells. *Ren Fail* **19**: pp 47-60.

Turnage RH, Guice K S and Oldham K T (1994) Pulmonary Microvascular Injury Following Intestinal Reperfusion. *New Horiz* **2**: pp 463-475.

Turnage RH, Kadesky K M, Bartula L, Guice K S, Oldham K T and Myers S I (1995) Splanchnic PGI2 Release and "No Reflow" Following Intestinal Reperfusion. *J Surg Res* **58**: pp 558-564.

Turnage RH, Kadesky K M, Myers S I, Guice K S and Oldham K T (1996) Hepatic Hypoperfusion After Intestinal Reperfusion. *Surgery* **119**: pp 151-160.

Uhlmann S, Uhlmann D and Spiegel H U (1999) Evaluation of Hepatic Microcirculation by in Vivo Microscopy. *J Invest Surg* **12**: pp 179-193.

Unal S, Demirkan F, Arslan E, Cin I, Cinel L, Eskandari G and Cinel I (2003) Comparison of Ischemic and Chemical Preconditioning in Jejunal Flaps in the Rat. *Plast Reconstr Surg* **112**: pp 1024-1031.

Ustundag B, Kazez A, Demirbag M, Canatan H, Halifeoglu I and Ozercan I H (2000) Protective Effect of Melatonin on Antioxidative System in Experimental Ischemia-Reperfusion of Rat Small Intestine. *Cell Physiol Biochem* **10**: pp 229-236.

Vachharajani TJ, Work J, Issekutz A C and Granger D N (2000) Heme Oxygenase Modulates Selectin Expression in Different Regional Vascular Beds. *Am J Physiol Heart Circ Physiol* **278**: pp H1613-H1617.

Van Leeuwen PA, Boermeester M A, Houdijk A P, Ferwerda C C, Cuesta M A, Meyer S and Wesdorp R I (1994) Clinical Significance of Translocation. *Gut* **35**: pp S28-S34.

Varela JE, Cohn S M, Giannotti G D, Dolich M O, Ramon H, Wiseberg J A and McKenney M (2001) Near-Infrared Spectroscopy Reflects Changes in Mesenteric and Systemic Perfusion During Abdominal Compartment Syndrome. *Surgery* **129**: pp 363-370.

Vejchapipat P, Proctor E, Ramsay A, Petros A, Gadian D G, Spitz L and Pierro A (2002) Intestinal Energy Metabolism After Ischemia-Reperfusion: Effects of Moderate Hypothermia and Perfluorocarbons. *J Pediatr Surg* **37**: pp 786-790.

Virlos IT, Inglott F S, Williamson R C and Mathie R T (2003) Differential Expression of Pulmonary Nitric Oxide Synthase Isoforms After Intestinal Ischemia-Reperfusion. *Hepatogastroenterology* **50**: pp 31-36.

Vlasov TD, Smirnov D A and Nutfullina G M (2002) Preconditioning of the Small Intestine to Ischemia in Rats. *Neurosci Behav Physiol* **32**: pp 449-453.

Wada K, Montalto M C and Stahl G L (2001) Inhibition of Complement C5 Reduces Local and Remote Organ Injury After Intestinal Ischemia/Reperfusion in the Rat. *Gastroenterology* **120**: pp 126-133.

Wang Q, Meyer T A, Boyce S T, Wang J J, Sun X, Tiao G, Fischer J E and Hasselgren P O (1998) Endotoxemia in Mice Stimulates Production of Complement C3 and Serum Amyloid A in Mucosa of Small Intestine. *Am J Physiol* **275**: pp R1584-R1592.

Warnecke HB, Schirmeier A, Nussler A K, Platz K P, Stange B, Nussler N C, Radke C, Neuhaus P and Mueller A R (2002) The Combined Treatment With L-Arginine and Methylprednisolone Improves Graft Morphology and Mucosal Barrier Function. *Transplant Proc* **34**: pp 996-998.

Weiss SJ (1989) Tissue Destruction by Neutrophils. *N Engl J Med* **320**: pp 365-376.

Williams JP, Pechet T T, Weiser M R, Reid R, Kobzik L, Moore F D, Jr., Carroll M C and Hechtman H B (1999) Intestinal Reperfusion Injury Is Mediated by IgM and Complement. *J Appl Physiol* **86**: pp 938-942.

Willis D, Moore A R, Frederick R and Willoughby D A (1996) Heme Oxygenase: a Novel Target for the Modulation of the Inflammatory Response. *Nat Med* **2**: pp 87-90.

Wilmore DW (1999) Growth Factors and Nutrients in the Short Bowel Syndrome. *JPEN J Parenter Enteral Nutr* **23**: pp S117-S120.

Wink DA, Hanbauer I, Krishna M C, DeGraff W, Gamson J and Mitchell J B (1993) Nitric Oxide Protects Against Cellular Damage and Cytotoxicity From Reactive Oxygen Species. *Proc Natl Acad Sci U S A* **90**: pp 9813-9817.

Wink DA and Mitchell J B (1998) Chemical Biology of Nitric Oxide: Insights into Regulatory, Cytotoxic, and Cytoprotective Mechanisms of Nitric Oxide. *Free Radic Biol Med* **25**: pp 434-456.

Wischmeyer PE (2002) Glutamine and Heat Shock Protein Expression. *Nutrition* **18**: pp 225-228.

Wolfard A, Szalay L, Kaszaki J, Sahin-Toth G, Vangel R, Balogh A and Boros M (2002) Dynamic in Vivo Observation of Villus Microcirculation During Small Bowel Autotransplantation: Effects of Endothelin-A Receptor Inhibition. *Transplantation* **73**: pp 1511-1513.

Wray S, Cope M, Delpy D T, Wyatt J S and Reynolds E O (1988) Characterization of the Near Infrared Absorption Spectra of Cytochrome Aa3 and Haemoglobin for the Non-Invasive Monitoring of Cerebral Oxygenation. *Biochim Biophys Acta* **933**: pp 184-192.

Wu B, Iwakiri R, Tsunada S, Utsumi H, Kojima M, Fujise T, Ootani A and Fujimoto K (2002) INOS Enhances Rat Intestinal Apoptosis After Ischemia-Reperfusion. *Free Radic Biol Med* **33**: pp 649-658.

Wu B, Ootani A, Iwakiri R, Fujise T, Tsunada S, Toda S and Fujimoto K (2004) Ischemic Preconditioning Attenuates Ischemia-Reperfusion-Induced Mucosal Apoptosis by Inhibiting the Mitochondria-Dependent Pathway in Rat Small Intestine. *Am J Physiol Gastrointest Liver Physiol* **286**: pp G580-G587.

Wu Y, Kudsk K A, DeWitt R C, Tolley E A and Li J (1999) Route and Type of Nutrition Influence IgA-Mediating Intestinal Cytokines. *Ann Surg* **229**: pp 662-667.

Xia G, Martin A E and Besner G E (2003) Heparin-Binding EGF-Like Growth Factor Downregulates Expression of Adhesion Molecules and Infiltration of Inflammatory Cells After Intestinal Ischemia/Reperfusion Injury. *J Pediatr Surg* **38**: pp 434-439.

Xia G, Martin A E, Michalsky M P and Besner G E (2002) Heparin-Binding EGF-Like Growth Factor Preserves Crypt Cell Proliferation and Decreases Bacterial Translocation After Intestinal Ischemia/Reperfusion Injury. *J Pediatr Surg* **37**: pp 1081-discussion.

Xiao F, Eppihimer M J, Young J A, Nguyen K and Carden D L (1997) Lung Neutrophil Retention and Injury After Intestinal Ischemia/Reperfusion. *Microcirculation* **4**: pp 359-367.

Yagihashi A, Tsuruma T, Tarumi K, Kameshima T, Yajima T, Yanai Y, Watanabe N and Hirata K (1998) Prevention of Small Intestinal Ischemia-Reperfusion Injury in Rat by Anti-Cytokine-Induced Neutrophil Chemoattractant Monoclonal Antibody. *J Surg Res* **78**: pp 92-96.

Yamaguchi T, Dayton C, Shigematsu T, Carter P, Yoshikawa T, Gute D C and Korthuis R J (2002) Preconditioning With Ethanol Prevents Postischemic Leukocyte-Endothelial Cell Adhesive Interactions. *Am J Physiol Heart Circ Physiol* **283**: pp H1019-H1030.

Yamamoto S, Tanabe M, Wakabayashi G, Shimazu M, Matsumoto K and Kitajima M (2001) The Role of Tumor Necrosis Factor-Alpha and Interleukin-1beta in Ischemia-Reperfusion Injury of the Rat Small Intestine. *J Surg Res* **99**: pp 134-141.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K and Masaki T (1988) A Novel Potent Vasoconstrictor Peptide Produced by Vascular Endothelial Cells. *Nature* **332**: pp 411-415.

Yang W, Koti R, Glantzounis G, Davidson B R and Seifalian A M (2003) Arterialization of the Portal Vein Improves Hepatic Microcirculation and Tissue Oxygenation in Experimental Cirrhosis. *Br J Surg* **90**: pp 1232-1239.

Yang ZJ, Bosco G, Montante A, Ou X I and Camporesi E M (2001) Hyperbaric O2 Reduces Intestinal Ischemia-Reperfusion-Induced TNF-Alpha Production and Lung Neutrophil Sequestration. *Eur J Appl Physiol* **85**: pp 96-103.

Yellon DM and Dana A (2000) The Preconditioning Phenomenon: A Tool for the Scientist or a Clinical Reality? *Circ Res* **87**: pp 543-550.

Younes M, Schoenberg M H, Jung H, Fredholm B B, Haglund U and Schildberg F W (1984) Oxidative Tissue Damage Following Regional Intestinal Ischemia and Reperfusion in the Cat. *Res Exp Med (Berl)* **184**: pp 259-264.

Zamir O, Hasselgren P O, Higashiguchi T, Frederick J A and Fischer J E (1992) Effect of Sepsis or Cytokine Administration on Release of Gut Peptides. *Am J Surg* **163**: pp 181-185.

Zhang W, Bain A and Rombeau J L (1995) Insulin-Like Growth Factor-I (IGF-I) and Glutamine Improve Structure and Function in the Small Bowel Allograft. *J Surg Res* **59**: pp 6-12.

Zhao H, Montalto M C, Pfeiffer K J, Hao L and Stahl G L (2002) Murine Model of Gastrointestinal Ischemia Associated With Complement-Dependent Injury. *J Appl Physiol* **93**: pp 338-345.

Ziegler TR, Estivariz C F, Jonas C R, Gu L H, Jones D P and Leader L M (1999) Interactions Between Nutrients and Peptide Growth Factors in Intestinal Growth, Repair, and Function. *JPEN J Parenter Enteral Nutr* **23**: pp S174-S183.

Zimmerhackl B, Parekh N, Brinkhus H and Steinhausen M (1983) The Use of Fluorescent Labeled Erythrocytes for Intravital Investigation of Flow and Local Hematocrit in Glomerular Capillaries in the Rat. *Int J Microcirc Clin Exp* **2**: pp 119-129.

Zingarelli B, Sheehan M and Wong H R (2003) Nuclear Factor-KappaB As a Therapeutic Target in Critical Care Medicine. *Crit Care Med* **31**: pp S105-S111.

Zou L, Attuwaybi B and Kone B C (2003) Effects of NF-Kappa B Inhibition on Mesenteric Ischemia-Reperfusion Injury. *Am J Physiol Gastrointest Liver Physiol* **284**: pp G713-G721.

Appendix 1. Publications and Presentations arising from the thesis

Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischemia Reperfusion Injury of the Intestine and Protective Strategies against injury. *Digestive Diseases and Sciences* 2004; 49; 9: 1359-1377.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischaemic preconditioning improves the microvascular perfusion and oxygenation following reperfusion injury of the intestine. *British Journal of Surgery* 2005; 92; 9: 1169-1176.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Effects of Ischemic Preconditioning on the Mucosal Villus Microcirculation in the Amelioration of Warm Ischemia Reperfusion Injury of the Intestine. *Microcirculation* 2005;12; 8: 615-625.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Pyrrolidine dithiocarbamate reduces ischaemia reperfusion injury of the small intestine. *World Journal of Gastroenterology* 2005 14; 11(46):7308-13..

Mallick IH, Yang W, Winslet MC, Seifalian AM. Pyrrolidine Dithiocarbamate Protects Small Bowel From Warm Ischemia Reperfusion Injury of The Intestine: The Role of Heme Oxygenase. (Submitted to *Shock*).

Published abstracts and presentations to learned societies

Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischemic preconditioning improves the intestinal oxygenation in reperfusion injury of the intestine.

J Vasc Res 2004; Suppl 2: 41: 54.

Oral Presentation at the European Microcirculation Society, Lisbon, Portugal, Sep 2004.

Mallick IH, Yang W, Winslet MC, Seifalian AM. The Role of Ischemic preconditioning on the intestinal microvascular perfusion and oxygenation in reperfusion injury of the intestine.

J Surg Res 2004; 121: 245.

Oral Presentation at the Association for Academic Surgery, Houston, Texas, USA, Nov 2004.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Effect of Ischaemic Preconditioning of the Intestine on the Portal Venous flow.

Endoscopy 2004; 36: 846.

Oral Presentation at the Irish Society of Gastroenterology Spring 2004 meeting, Kilkenny, Ireland, Jun 2004.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Pyrrolidine improves intestinal microvascular perfusion and tissue oxygenation in ischemia reperfusion injury of the small bowel.

J Gastro Intest Surg 2004; Suppl 1: 8 (7); A 287.

Poster Presentation at the International Society for Digestive surgery, Yokohama, Japan, Dec 2004.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Effects of Ischemic Preconditioning on the Mucosal Villus Microcirculation following Ischemia Reperfusion Injury of the Intestine and its relationship with heme oxygenase. *Gastroenterology* Suppl Apr 2005; Suppl 2:128(4): A657.
Poster Presentation at the Digestive Diseases Week, Chicago, Illinois, USA, May 2005 (**Poster of Distinction**).

Mallick IH, Yang W, Winslet MC, Seifalian AM. Role of Ischaemic Preconditioning on the Mucosal Villus Microcirculation in the Amelioration of Ischaemia Reperfusion Injury of the Intestine.
Poster Presentation at the Surgical Academic Research Society, Newcastle, Jan 2005

Mallick IH, Yang W, Winslet MC, Seifalian AM Effect Of Ischaemic Preconditioning On The Intestinal Intracellular Tissue Oxygenation During Ischaemia Reperfusion Injury Of The Intestine. Gut 2005; Suppl 54: A103.
Poster Presentation at the British Society of Gastroenterology, Birmingham 2005.

Mallick IH, Yang W, Winslet MC, Seifalian AM. Effect of Ischaemic Preconditioning on the intestinal microcirculation.
Microcirculation 2004; 11(6): 527-558.
Oral Presentation at the 41st Annual Spring Meeting, British Microcirculation Society, Sheffield, Apr 2004

Mallick IH, Yang W, Winslet MC, Seifalian AM. Effect of Pyrrolidine dithiocarbamate on intestinal microvascular perfusion in ischaemia reperfusion injury of the small bowel. Br J Surg 2005; 92: Suppl. 1: 83.

Poster Presentation at the Annual Meeting of the Association of the Surgeons of Great Britain and Ireland, Glasgow 2005.

Appendix 2. Protocol for Fluorescence Labelling of RBC

Stock Solution 1 (adjust to pH 7.4)

Barbital Sodium	2.55 g
1M HCl	10 ml
NaCl	6.8 g
Bring to	0.5 L

Stock Solution 2:-

$MgSO_4.7H_2O$ 24.6 g

100ml distilled water makes 1M solution

Stock Solution 3(0.03M $CaCl_2$)**:**

$CaCl_2.2H_2O$	4.41 g

100ml distilled water makes solution.

Note: *Stock solutions can be kept for around a month in a fridge at* 4^0C.

Working Solution (glucose saline buffers)

Stock 1	50 ml
Stock 2	0.1 ml
Stock 3	0.1 ml
Glucose	4.2 g

Make up to 200ml with distilled water

FITC for red blood cells:-

40mg of FITC

2ml of glucose saline buffer

Labelling cells:

1. Collect approx 8ml blood in heparinised tube or as much as you need.

2. Centrifuge blood at 400g or 2000r.p.m for 10mins

3. Remove plasma + buffy coat

4. Wash cells with glucose saline buffer 5 times

5. 1ml of washed red cells are added to 1ml of buffer and 0.4ml of FITC

6. Leave for 1.5 to 2hrs at room temp

7. Labelled cells are washed 3 times until no colouring is left in supernatant

8. Suspend in glucose saline buffer in 1:2 dilution.